Feeding ecology of whale sharks at Ningaloo Reef, Western Australia

by

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Institute for Marine and Antarctic Studies (IMAS)
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Statements and declarations

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Signed,

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June 2017

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General abstract

The largest fish in the world, the iconic whale shark, is a highly migratory species of high conservation risk, that seasonally aggregates at predictable coastal locations in tropical and temperate regions around the world. A number of logistical challenges, however, have hindered our ability to understand key aspects of their ecology, which is imperative in assisting management efforts. In the last decades, biochemical approaches offer tools to identify patterns and drivers of the feeding habits of marine animals at large temporal (from weeks to seasons) and spatial (regional to ocean basin) scales. In this thesis, the feeding ecology of whale sharks were investigated using signature fatty acid and stable isotope analyses of whale shark sub-dermal tissue and a wide range of putative prey species including zooplankton and micronekton collected at Ningaloo Reef, Western Australia.

Fatty acid analysis of whale shark sub-dermal tissue indicated large intraspecific variability in diet, with the differences observed likely due to individual specialization and also changes in the primary and secondary production between years of sampling. Significant differences were found between fatty acid profiles of whale sharks and their potential prey collected at Ningaloo Reef, the latter showing signatures typical of pelagic systems. High relative levels of the omega-6 long-chain (≥ C_{20}) polyunsaturated fatty acid (LC-PUFA) - arachidonic acid (20:4ω6) - in whale shark profiles suggested that these animals have a wide foraging range with important contribution to their diet from benthic and deep-water habitats including demersal zooplankton.

An experiment assessed the appropriate treatment of sub-dermal tissue and the prey species samples prior to stable isotope analysis. Failing to remove known sources of isotopic variability (lipids, urea or inorganic carbonate) from samples can potentially confound the reconstruction of food webs and movement patterns. Lipid extraction by chloroform:methanol:water and removal of urea by rinsing with deionized water was recommended as a means to standardize δ^{13}C and δ^{15}N values in whale shark sub-dermal tissue, and elasmobranchs in general. For the analysis of δ^{13}C values of zooplankton and micronekton, an aliquot of the sample should be lipid extracted and acidified, with the acidification step employed only if inorganic carbonate is present. In contrast, the erratic effect of lipid extraction and removal of inorganic carbonate in δ^{15}N values in zooplankton and micronekton, indicated the need for an untreated sub-sample to be used for the analysis of nitrogen. Previous published mathematical normalizations used to adjust isotopic values were not suitable for all the taxa and tissue types in this study.

Based on stable isotope analysis, whale sharks have an estimate trophic position of 2.6. Enriched ^{13}C signatures in whale sharks compared to collected prey, suggested that these large elasmobranchs are highly reliant on inshore benthic food webs, similar to findings observed in the signature fatty acids. Size- and sex-specific habits of feeding among whale sharks are revealed. Values of $\delta^{15}N$ increased with size of whale sharks, indicating they consume higher trophic level prey as they age. An ontogenetic transition in habitat from offshore to more coastal habitats as whale shark grows (from 3 to 8 m) was also observed.

By improving the applicability and ecological interpretation of non-invasive biochemical approaches, this study has advanced our understanding of the feeding ecology of whale sharks, and has in turn set the path for future studies to use these cost-effective techniques in other aggregations. Given that global populations of whale sharks are in decline, information provided in this study will assist in the development of conservation and management strategies to ensure connectivity for this highly mobile elasmobranchs.

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Chapter 1

General introduction, aims and thesis structure

1.1. General Introduction

1.1.1. The whale shark: a high-risk conservation species

The world's largest fish (> 12 m), the whale shark (*Rhincodon typus*, Smith 1828), is a wide-ranging filter-feeder that occurs throughout the world's tropical and warm-temperate oceans (Rowat and Brooks, 2012). Globally there are two subpopulations of whale sharks one in the Indo-Pacific and other in the Atlantic Ocean (Vignaud et al. 2014). Highly migratory in nature, whale sharks can travel thousands of kms (e.g. Wilson et al. 2006, Sleeman et al. 2010, Hueter et al. 2013) between oceanic and coastal habitats. In some nearshore locations around the world, predictable seasonal aggregations are known to occur including in north-western Australia, Mozambique, Philippines, Galapagos, Seychelles, Djibouti and Mexico (Rowat and Brooks, 2012). It is believed that *R. typus* aggregate in these areas to take advantage of peaks in prey abundance (e.g. Clark and Nelson, 1997, Heyman et al. 2001, Jarman and Wilson, 2004, Meekan et al. 2009) and that their movements can be correlated with parameters including geostrophic currents, sea surface temperature and local bathymetry (Wilson et al. 2001b, Sleeman et al. 2007, 2009).

Aggregations of whale sharks are often the focus of ecotourism that make major contributions to local economies and contribute valuable data about the structure of whale shark populations (Meekan et al. 2006). Whale sharks face numerous anthropogenic threats including targeted fisheries, by-catch and boat-strikes. There is now evidence of a decline of whale shark populations across much of their range (Pierce and Norman, 2016). Whale sharks share the typical elasmobranch features of slow growth rates, late maturity and extended longevity (Colman, 1997). These k-selected life history traits make them even more vulnerable to exploitation (Bradshaw et al. 2008). From the evidence of recent population declines, the species is considered 'Endangered' under the IUCN Red List Criteria and it is also listed in the Convention on International Trade in Endangered Species and in the Convention of Migratory Species.

Protection of highly mobile animals such as whale sharks requires a good understanding of the species' spatio-temporal ecology (Simpfendorfer et al. 2011). Knowledge of feeding behavior throughout all life history stages are key for the successful development and implementation of management and conservation strategies (Hussey et al. 2012).

1.2.1. Knowledge of whale shark feeding ecology

The whale shark is one of the three extant sharks that filter-feed. Other species include the megamouth (*Megachasma pelagios*) and basking (*Cetorhinus maximus*) sharks. There is little doubt that the seasonal occurrence of whale shark aggregations at some locations is linked to feeding on ephemeral, but predictable prey concentrations (Heyman et al. 2001, Wilson et al. 2001a). However, how *R. typus* locate these feeding opportunities, is still largely unknown. Most whale shark aggregations are comprised of juvenile males indicating the likely size and sex segregation in habitat and diet (Rowat and Brooks, 2012). Different feeding mechanisms have been observed in whale sharks, such as ram-feeding, suction-feeding and a combination of both, that enable them to successfully target high-density patches of food (Nelson and Eckert, 2007, Taylor 2007). Dietary studies of whale shark aggregations have reported a wide range of prey, most of which are pelagic invertebrates and small bait fish, including fish spawn in Belize (Heyman et al. 2001), crab larvae in Christmas Island (Meekan et al. 2009), copepod blooms in La Paz, Mexico (Clark and Nelson, 1997), krill schools in Ningaloo Reef, Western Australia (Jarman and Wilson, 2004) and anchovies off New Zealand (Duffy 2010). However, these studies are based on anecdotal observations of feeding events and the analysis of stomach contents and faeces, which can bias the importance of some prey items and also only reflect dietary input at short scales of time and space (hours to days, m to km) (Iverson 2009).

Studies using satellite tags have revealed large scale (> 1000s km) migrations for whale sharks and highlighted their ability to dive to meso and bathypelagic depths (Wilson et al. 2006, Sleeman et al. 2010, Hueter et al. 2013). Although the reasons for these movements are still unclear, they are likely to be foraging related (Meekan et al. 2015). Alternative approaches are thus needed to elucidate the feeding ecology of whale sharks in environments where they are difficult to observe, for example, when they are large distances from the coast or at depth.

1.3.1. Biochemical approaches: fatty acids and stable isotope as trophic markers

In the last decades, biochemical techniques, including fatty acids (FA) and stable isotope (SIA) analyses, have been developed as non-lethal tools to investigate the feeding ecology of highly migratory animals at a variety of temporal and spatial scales (e.g. Hobson et al. 1997, Budge et al. 2006, Graham et al. 2010, Bowen and Iverson, 2013). These techniques are based on the assumption 'you are what you eat', meaning that trophic markers of prey (e.g. long chain, ≥ C_{20}, polyunsaturated FA (LC-PUFA) or carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes) are deposited in a predictable manner into tissues of a consumer, thus providing an assimilated record of diet and not just recently ingested food (Dalsgaard et al. 2003, Newsome

et al. 2010). Since the FA and isotopic composition of food webs vary temporally (inter-annual or seasonal) and spatially (offshore/inshore or pelagic/benthic) depending on localized biogeochemical processes (e.g. Montoya 2007, Budge et al. 2008), consumer's tissues composition will then reflect those particular habitats and environments where the animal has foraged and resided for a certain period of time (Iverson 2009, DeNiro and Epstein, 1978, 1981).

To interpret data from biochemical approaches, the incorporation time of trophic markers or turnover rates of the tissues being analysed must be considered. Turnover rates vary among tissues, with faster rates in highly active metabolic tissues such as blood, plasma and liver, somewhat slower rates in muscle, and even slower rates in structural tissue such as bone or cartilage (MacNeil et al. 2006, Beckmann et al. 2013). Although muscle is the tissue used most commonly for analysis in elasmobranchs, biopsies of sub-dermal tissue also have the potential to be used for FA and SI analysis. Sub-dermal tissue is useful because it can be taken via external biopsy sampling which is the least invasive for the animal. Biopsy samples of sub-dermal tissue is highly reproducible and only a small amount of tissue is needed for analysis (< 0.5 g) (Couturier et al. 2013 a,b, Rohner et al. 2013).

To date, biochemical analysis has been used in only a few studies to investigate the diet, trophic ecology and movement patterns of whale sharks. In Mozambique, Western Indian Ocean, analysis of lipids and FA of sub-dermal tissue suggested a major contribution from meso- and bathypelagic sources to whale shark diet (Couturier et al. 2013b, Rohner et al. 2013). In contrast, values of δ^{13}C and δ^{15}N in muscle of whale sharks from the Arabian Sea, in India, were characteristic of a diet based on pelagic zooplankton. In addition, ontogenetic shifts in diet were observed with an increasing contribution of prey at higher trophic levels as whale sharks moved from the open ocean to coastal habitats (Borrell et al. 2011 a,b). These results implied, for the first time, a wider foraging range for whale sharks. These sharks may be foraging in benthic and deep-water habitats and they may display size, and possibly sex segregation in diet and habitat.

Despite these recent studies providing new insights into the feeding ecology of whale sharks, it remains unknown whether the observed foraging and movement behaviors are exclusively of those aggregations in the western Indian Ocean, or are a common trait of the species. Collection of samples from individuals from a range of locations of different sex, size, maturity stages and, when possible, from different years, should be the target of future studies in order to document ontogenetic shifts, habitat segregation, individual specialization and temporal changes in dietary and habitat use. Data produced from such studies would inform

conservation and management strategies of whale shark populations world-wide. Although previous studies have established the foundation for the use of biochemical approaches in other aggregations, there is also a need to further understand and interpret FA and SIA data and the associated tissue being analysed.

1.4.1. Limitations of biochemical approaches

Application of biochemical approaches must consider various parameters including (1) the dynamics of FA and isotopic incorporation (turnover rates) in animal tissues, (2) the diet-tissue discrimination factors (DTDFs) for stable isotopes of carbon and nitrogen and (3) sample preparation for SIA (Budge et al. 2006, Wolf et al. 2009). Quantification of turnover rates and DTDFs requires controlled feeding studies in the laboratory, which for large elasmobranchs are logistically and ethically difficult to undertake. As a result, most studies use information published on small species that can be held in aquaria.

Critical to the interpretation of SIA results is the appropriate treatment of samples before analysis. It is well-recognized that lipids and inorganic carbonate content in samples can bias δ^{13}C values leading to misinterpretation of carbon sources in food webs, and thus these components of samples need to be removed where possible (Post et al. 2007, Logan et al. 2008, Pomerleau et al. 2014). However, there is still no widely adopted standardized sample treatment protocols. Methods are often applied in an inconsistent manner across studies, species and tissues. In elasmobranchs, high concentrations of nitrogenous waste in tissues can further alter δ^{15}N values leading to a downward bias in estimates of trophic level. Recent studies suggested that urea, in addition to lipids, need to be removed from elasmobranch tissues (Churchill et al. 2015, Li et al. 2016). Further experimental work is needed to investigate the effects of lipids, inorganic carbonate and urea in elasmobranchs and their potential prey in order to properly interpret food web interactions. The lack of standardized protocols constitutes a barrier for the comparison of SIA results within and among studies.

1.5.1. Whale shark aggregation at Ningaloo Reef, Western Australia: study site

Ningaloo Reef, in north-western Australia, is the longest fringing reef system in Australia stretching 290 kms (Cassata and Collins, 2008). Listed as a World Heritage area, much of the Ningaloo Reef is within the boundary of the Ningaloo Reef Marine Park offering protection to its high species diversity including marine megafauna such as marine mammals, whales and sharks. Seasonal aggregations of whale sharks around 300 - 500 sharks occur at Ningaloo Reef (Andrzejaczek et al. 2016) every year between March and June (Fig. 1.1). This

aggregation consists mainly of immature males (Meekan et al. 2006), suggesting that the region may be important for feeding, rather than for mating or breeding (Norman and Stevens, 2007). The occurrence of whale sharks in the area in the austral autumn has been linked to an increase in productivity adjacent to the reef, which is triggered by an increase in oceanic nutrients (Wyatt et al. 2012a). As a result, the intensification in productivity leads to a great increase in the biomass of zooplankton, in particular the euphausiid, *Pseudeuphausia latifrons*, which is thought to be one of the main food sources of whale sharks at Ningaloo Reef (Wilson et al. 2003, Jarman and Wilson, 2004). Oceanographic and physical signals may also influence the abundance of whale sharks at Ningaloo Reef. During La Niña years, the strengthening of the Southern Oscillation Index and the off-shore Leeuwin Current, which flows south, potentially brings a greater number of whale sharks to Ningaloo Reef (Wilson et al. 2001, Sleeman et al. 2010b).

A successful ecotourism industry has developed around the seasonal aggregation of whale sharks at Ningaloo Reef, which attracts thousands of participants every year, yielding revenues of millions of dollars to the local community (Catlin et al. 2010). Satellite tagging studies have shown that whale sharks departing Ningaloo move north towards Indonesia and the Timor Sea (Wilson et al. 2006, Sleeman et al. 2010). A decline in shark abundance and size in the Ningaloo aggregation has been linked to fishing pressure in South East Asia, where they are targeted for their fins and flesh (Bradshaw et al. 2008). Information on the feeding habits including diet, trophic ecology, related movement patterns and foraging ranges of these whale sharks at different spatial and temporal scales is pivotal to ensure the conservation of this highly mobile elasmobranch.

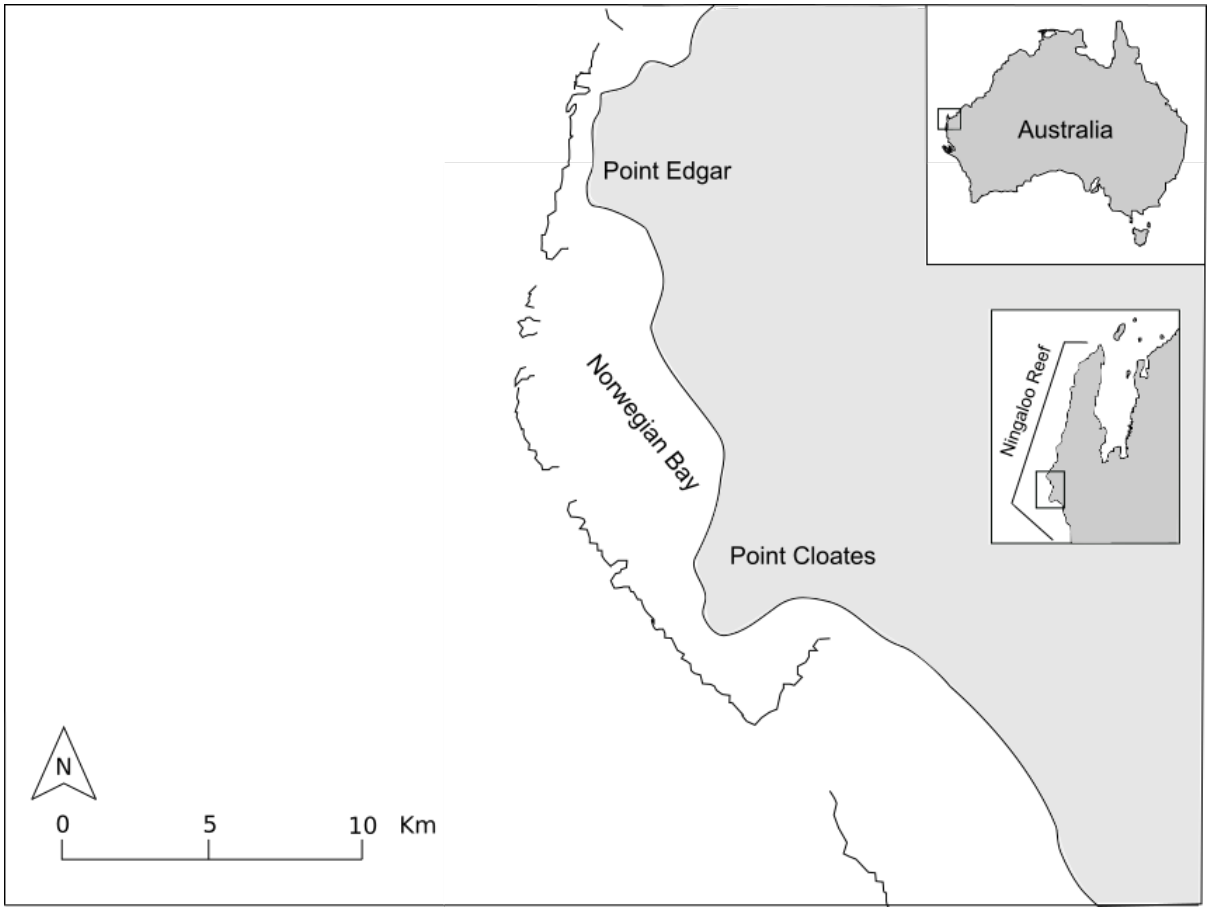

Figure 1.1. Study area at Ningaloo Reef, Western Australia. Beyond the shore (shaded area) the seaward edge of the reef is shown. Whale sharks occur to the west of this reef edge.

1.6.1. Aims and structure of this study

The overall aim of this thesis was to investigate the feeding ecology of whale sharks aggregating at Ningaloo Reef. Applying biochemical methods including fatty acid and stable isotope analysis, has provided information on feeding habits at large temporal and spatial scales. The results of this work are reported in three research chapters that have been prepared as stand-alone manuscripts and have been published or are in preparation for submission to scientific journals.

Chapter 2: investigated the diet and foraging ranges of whale sharks using lipid and fatty acid analysis of their sub-dermal tissue and putative potential prey sampled at Ningaloo Reef.

Chapter 3: developed standardized protocols for treatment of whale shark sub-dermal tissue and its potential prey prior to SIA, to better assist in the interpretation of results.

Chapter 4: examined the trophic ecology and movement patterns of whale sharks using stable isotope analysis of whale shark sub-dermal tissue and its putative potential prey sampled at Ningaloo Reef.

A general discussion (**Chapter 5**) summarizing the main findings and implications of this thesis and provide recommendations for future research directions is also presented.

Chapter 2

Intraspecific variability in diet and implied foraging ranges of whale sharks at Ningaloo Reef, Western Australia, from signature fatty acid analysis

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Abstract

We examined the feeding ecology of whale sharks by analyzing the signature fatty acids of their sub-dermal tissue and those of an extensive set of potential prey collected at Ningaloo Reef, Western Australia in 2013, 2014 and 2015. Sub-dermal tissue of whale sharks was low in lipid content (4.0 mg g^{-1} dry mass) and dominated by phospholipids (72% of total lipid) with a calculated energy density of 18.7 kJ g^{-1} dry mass. There was significant intraspecific variability in fatty acid profiles of whale sharks, with cluster analysis identifying four distinct groups in 2013 and five groups in 2014. As this variability was not related to sex or size-class, we suggest that it may be attributed to differences in the feeding habitats used by these groups of whale sharks. Variation in dietary patterns was also observed between years likely due to changes in the primary and secondary producers. Examination of food web interactions showed that fatty acid profiles of whale sharks and their presumed prey were significantly different, suggesting that sharks fed over a wider range of habitats, including deep waters. Our findings show that signature fatty acids of sub-dermal tissue can be used to examine broad trophic pathways and to identify spatial and temporal changes in diet of these large and wide-ranging animals.

2.1. Introduction

The whale shark (*Rhincodon typus* Smith 1828) is the largest of the filter-feeding sharks and inhabits tropical and sub-tropical oceans worldwide (Ebert et al. 2013). As adults, these animals mostly reside in the open ocean where they are thought to follow zooplankton prey that undergoes diel vertical migrations between depths of 200 - 500 m during the day and in the surface (0 - 100 m) at night (Meekan et al. 2015). Because of the remoteness of these oceanic habitats, studies of the feeding behavior of these sharks are very challenging. However, predictable aggregations of whale sharks sometimes occur on tropical coasts, offering an opportunity to examine their ecology and diet in habitats more easily accessible to researchers.

Evidence from a number of studies at aggregation sites such as Ningaloo Reef, Western Australia (Meekan et al. 2006), Belize (Heyman et al. 2001) and Christmas Island (Meekan et al. 2009) suggest that whale sharks congregate in coastal habitats to target local pulses of prey availability (Colman, 1997, Compagno, 2001). A wide range of planktonic and nektonic organisms including copepods, gelatinous zooplankton (such as salps, siphonophores and jellyfish), chaetognaths, krill, mysids, amphipods, sergestids, fish eggs, small fish, shrimp and crab larvae have been identified as whale shark prey (see review Rowat and Brooks, 2012). Most reports of diet are based either on anecdotal observations of whale sharks feeding in coastal surface waters during the day, plankton tows (Clark and Nelson, 1997, Heyman et al. 2001, Nelson and Eckert, 2007, Taylor 2007, Motta et al. 2010, Ketchum et al. 2013, Robinson et al. 2013, Rohner et al. 2015) or stomach (e.g. Silas and Rajagopalan, 1963) and faecal (Jarman and Wilson, 2004, Meekan et al. 2009) analyses. However, these methods have some well-recognized drawbacks as they can overestimate the importance of some prey and only represent 'snapshots' of recent feeding events (Iverson 2009). For these reasons, a more holistic approach to examine temporal and spatial patterns in the feeding habits of these sharks is required (Iverson 2009).

In the last decade, signature fatty acid (FA) analysis has proved to be a useful tool to investigate the feeding ecology of elasmobranchs (rays, skates and sharks) (Pethybridge et al. 2011, McMeans et al. 2013, Wai et al. 2012, Pethybridge et al. 2014), and other free-ranging marine animals (e.g. Budge et al. 2008). When compared to other methods such as stomach content analyses, description of signature FA can provide longer term (weekly to monthly) dietary information (Budge et al. 2006, Iverson 2009, Beckmann et al. 2014). This is possible because some FA in animal tissues (e.g. long-chain, $\geq C_{20}$, polyunsaturated FA, LC-PUFA) can be used as biomarkers (also termed signature fatty acids here) as they pass

relatively unchanged from the low trophic levels, where they are biosynthesized, up the food chain (Dalsgaard et al. 2003). Analysis of FA not only allows food web interactions to be described, but also provides an assessment of spatial and temporal changes in the diet of predators, both among and within individuals or populations (Iverson 2009). The technique also has the advantage that only a small amount of tissue (< 0.5 g) is required for analysis, which can be removed as tissue biopsies from live animals without causing serious harm (Budge et al. 2006, Couturier et al. 2013a). This makes the technique ideally suited for examining the diets of protected species such as whale sharks that are not the subject of industrial fisheries and strand only very occasionally (Speed et al. 2009).

Recently, Couturier et al. (2013b) and Rohner et al. (2013) used signature FA of sub-dermal tissue to examine diets of whale sharks from an aggregation in Mozambique, western Indian Ocean. These studies indicated that whale sharks had a wider foraging range than previously suggested, with important contributions from meso- and bathypelagic sources including deep fish, macrozooplankton and demersal zooplankton. However, it remains unknown if these results characterize the diet of whale sharks over the wider Indian Ocean. Here, we investigate the feeding ecology of whale sharks sampled in the eastern Indian Ocean at Ningaloo Reef, Western Australia. We analyzed FA profiles of sub-dermal tissue of whale sharks collected in 2013 and 2014, and an extensive range of potential prey including zooplankton, small fishes and fish larvae, cephalopods, annelids, crab larvae, decapods, isopods, krill, mysids, amphipods and algae collected from 2013 – 2015. Fatty acid profiles were then used to assess and describe likely food web linkages and to investigate intra-population differences in diet associated with collection time, sex or size class of whale sharks from Ningaloo Reef. We also further examined the chemical composition (water and lipid content and lipid class determination) of whale shark sub-dermal tissue in an attempt to elucidate energy uptake, nutritional condition and resource usage patterns.

2.2. Material and methods

2.2.1. Collection of samples

Samples were collected during May of 2013, 2014 and 2015 at Ningaloo Reef, Western Australia (22° 33′ 45″ S, 113° 48′ 37″ E; Fig. 2.1), a time that coincided with the annual aggregation of whale sharks at this locality which is estimated to last from March to July every year. This study was conducted under approval from the University of Tasmania Animal Ethics Committee (A13102). Field work was carried out under permits and exemptions from the Department of Parks and Wildlife (SF009814, SF009227) and the Western Australian Department of Fisheries (2255, 2307).

2.2.1.1. Whale shark sub-dermal tissue

A total of 52 biopsies (19 in 2013 and 33 in 2014), were collected from different individual whale sharks. The presence or absence of claspers on the pelvic fins was used to identify the sex of an individual. Estimated total length (TL) of whale sharks ranged from 3 to 8.5 m and sharks were categorized in four groups according to size class: < 4 m, 4 - 6 m, > 6 - 8 m and > 8 m. A snorkeler extracted a sub-dermal tissue sample using a modified hand spear with a biopsy probe tip. A small core approximately 2 cm in length of sub-dermal tissue was taken from the left side of the shark behind the 1st dorsal fin. Immediately after collection, biopsies were cut in three equal parts in a transverse section in 2013 and in a longitudinal section in 2014. A third of the biopsy was stored frozen in liquid nitrogen for lipid content and FA analyses. In order to assess the distribution of FA along the sample of sub-dermal tissue, five whole biopsies were also collected in 2014 and divided into two equal parts (outer, closest to the skin and inner) prior to freezing.

2.2.1.2. Potential prey

Zooplankton

In 2013, a zooplankton survey was carried out offshore from the reef break of Norwegian Bay, between Point Edgar and Point Cloates, to examine any selective feeding by whale sharks related to depth (Fig. 2.1). This survey consisted of three cross-shelf transects (north, middle and south). Four to seven stations were sampled along each transect. Depths of stations varied according to the bathymetry of the area and were divided into: surface (from 0 to 2 m), upper (from surface to a maximum depth of 50 m) and bottom (from mid water column to

seafloor at a maximum depth of 90 m) layer. In 2014 only the surface layer was occasionally sampled (Fig. 2.1). Two types of nets were used to collect zooplankton. Firstly, a ring net with 200 µm mesh was used in surface tows. The second, used to sample deeper water layers, was a specially designed 300 µm mesh net that closed and opened at the desired depth by a 'choking' system. During all hauls, nets were towed for 10 minutes at a speed of ~2 knots. When retrieved, nets were rinsed with seawater and the plankton concentrated in the cod-end were transferred to 500 ml plastic jars and kept in insulated containers of seawater for transportation to shore. Once onshore, zooplankton samples were split in two using a Folsom's Sample Divider. Half of each sample was kept frozen for lipid content and FA analyses. Of the remaining half, a quarter of the sample was fixed with 70% ethanol in filtered seawater for identification of component plankton and a quarter for stable isotope analysis (results to be reported elsewhere). In addition, all samples were fractionated by filtering through 100, 300, 500 and 1000 µm sieves to assess possible selective feeding behavior by whale sharks related to prey size. When large (> 1000 µm) zooplankton was abundant in collections (for example cladocerans, salps, pyrosomes, chaetognaths, siphonophores, ctenophores (beroe) and other jellyfish), a few representatives of each species were frozen. Individuals of these taxa were pooled between years into the category of larger zooplankton due to low sample size.

Other invertebrates and small fish

We deployed single-chamber light traps (see Meekan et al. 2001 for design) to target mobile organisms (e.g. krill, cephalopods and small fishes) that were likely to avoid nets (Wilson et al. 2003). In 2013, we deployed two light traps (max. water column depth 7 m) in the inner part of the fringing reef just in front of Norwegian Bay (Fig. 2.1). Two traps were also deployed in the outer side close to the crest of the reef (max. water column depth 27 m). In 2014 and 2015, a total of twelve and four traps were deployed seawards of the outer side of the reef, respectively (max. water column depth 70 m) (Fig. 2.1). Light traps were deployed overnight and were suspended by floats so that the entrance to the light chamber was approximately 1 m below the surface. Samples collected by the traps were retrieved the next morning and transported to shore in insulated containers of seawater. Samples were then immediately sorted to the highest taxonomic resolution possible and frozen. Small fish (0 – 15 cm total length, TL) were frozen whole once euthanized, whereas large fish (> 15 cm TL) were sliced and blended. A homogenized subsample was used for lipid class and fatty acid analyses. Both phytoplankton and the macro-algae *Sargassum* sp. were also collected for lipid and FA analysis in 2013. For phytoplankton, surface water samples were collected with clean plastic buckets, and at a depth of 15 m with Niskin bottles. Samples were transported to shore and

immediately filtered onto 0.22 µm Whatman GF/F glass fiber filters and frozen. The *Sargassum* sp. was sampled opportunistically from seaweed that was collected by zooplankton tows, transported to shore in insulated containers of seawater, then sorted and frozen.

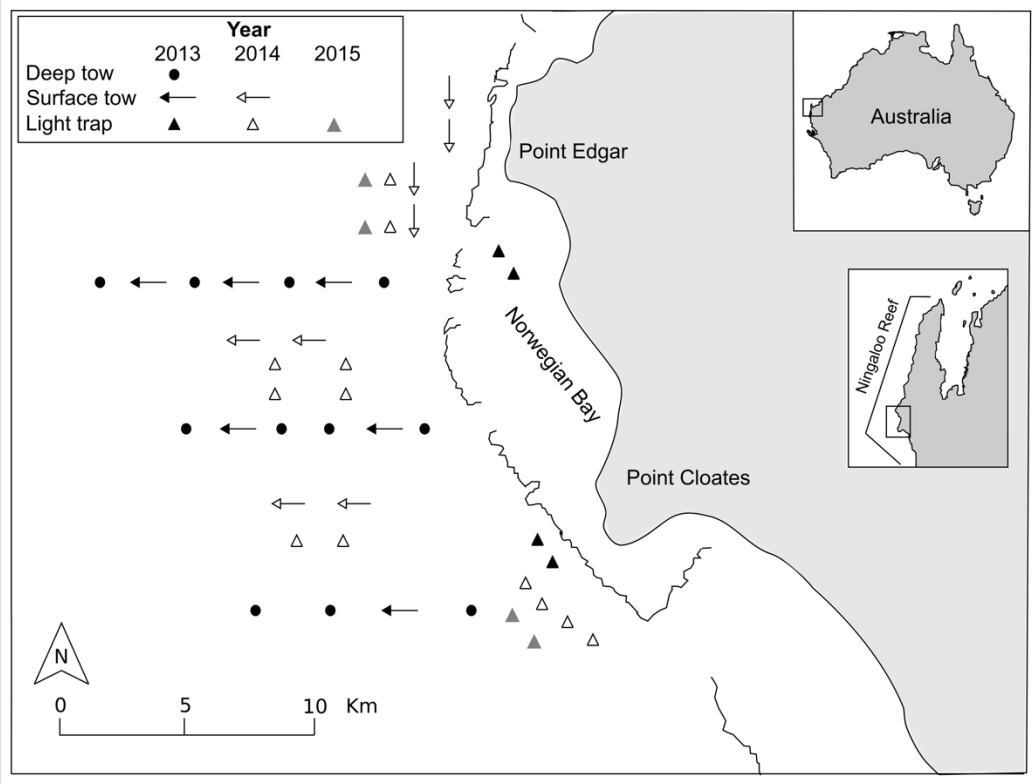

Figure 2.1. Map showing collection of potential prey at Ningaloo Reef (Western Australia) in May 2013, 2014 and 2015.

2.2.2. Lipid class and fatty acid analyses

2.2.2.1. Lipid extraction

Lipid and FA signature analyses was conducted at the CSIRO Marine Laboratories in Hobart, Tasmania, Australia. All samples were freeze-dried and weighed prior to analysis. Lipids were extracted following a modified Bligh and Dyer (1959) method using a one-phase methanol:chloroform:Milli-Q water (2:1:0.8 v/v/v) overnight extraction. In some instances, chloroform was substituted with dichloromethane. The following morning, the phases were separated by adding 10 ml of chloroform and 10 ml of saline Milli-Q water to reach a final ratio of chloroform:methanol:water of 1:1:0.9 v/v/v. The lower layer was retained and lipids recovered by the removal of solvents *in vacuo* using a rotary evaporator at ~40°C. The total lipid extract (TLE) was concentrated to dryness in 1.5 ml glass vials under a stream of inert

nitrogen gas and weighed. Samples were re-dissolved in chloroform and stored at -20°C for further analysis.

2.2.2.2. Lipid class determination

TLE samples were spotted in duplicate onto silica gel SIII Chromarods (5 µm particle size) using 1 µl disposable micropipettes along with standard solutions containing known quantities of common lipid classes including wax esters (WE), hydrocarbons (HC), triacylglycerols (TAG), free fatty acids (FFA), sterols (ST) and phospholipids (PL). Chromarods were developed in a polar solvent system (60:1:0.1 v/v/v, hexane:diethyl-ether:acetic acid) for 25 min and then dried in an oven for 10 min at 100°C. After drying, samples were immediately analysed with an Iatrascan Mark V TH10 thin layer chromatograph (TLC) with a flame ionization detector (FID). Peaks were identified by comparison of sample retention times in relation to the standards and peak areas quantified using SIC-480II Iatroscan^{TM} Integrating Software v.7.0-E (System Instruments Co., Mitsubishi Chemical medicine Corp., Japan). The FID was calibrated for each lipid class (phosphatidylcholine, cholesterol, cholesteryl oleate, oleic acid, squalene, TAG (derived from fish oil), wax ester (derived from orange roughy, *Hoplostethus atlanticus*, oil) and diacylglyceryl ether (DAGE; derived from shark liver oil; 0.1-10 µg range). Using predetermined linear regressions, peak areas were transformed to mass per µl spotted.

2.2.2.3. Fatty acid analyses

An aliquot of the TLE was trans-methylated to produce fatty acid methyl esters (FAME). FAME were obtained by adding 3 ml of methanol: hydrochloridric acid: chloroform (10:1:1, v/v/v) to an aliquot of the TLE and heated for 2 h at ~100°C. After cooling, 1 ml of Milli-Q water was added and FAME were extracted 3 times with 1.8 ml of hexane:dichloromethane (4:1, v/v). The FAME-extracted fraction was blown down under a gentle stream of nitrogen gas to dryness and an internal injection standard (C_{19} FAME or C_{23} FAME) added. FAME were analysed by gas chromatography (GC) using an Agilent Technologies 7890B GC equipped with a non-polar Equity^{TM} -1 fused silica capillary column (15 m x 0.1 mm internal diameter, 0.1 µl film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler. Helium was the carrier gas. Samples were injected in split-less mode at an oven temperature of 120°C, which was raised to 270°C after injection at 10°C min^{-1} and finally to 300°C at 5°C min^{1}. Agilent Technologies ChemStation software (Palo Alto, California, USA) was used to quantify FAME peaks. Identification of selected FA samples was further confirmed by GC-mass spectrometry (GC-MS) using a Finnigan ThermoQuest GCQ GC-MS

system (Finnigan, San Jose, California) fitted with an on-column injector and using Thermoquest Xcalibur software.

2.2.3. Water content and energy densities of whale shark sub-dermal tissue

Whale shark sub-dermal tissue was freeze-dried for 48 h, and the water fraction (WF) and wet/dry mass ratio determined by taking weights before and after drying. From lipid fraction values (LF) of dry mass (dm) and wet mass (wm), we calculated the proportion that consisted of protein (P) and carbohydrate (C) according to: (1) $PCF_{dm} = 1 - LF_{dm}$ and (2) $PCF_{wm} = 1 - LF_{wm} - WF$. Then, energy density of whale shark tissue was determined with the following equations and using published calorific values of 39.9 kJ g^{-1} for lipids and 17.8 kJ g^{-1} for protein and carbohydrates (Schmidt-Nielsen 1997), after Pethybridge et al. (2014):

(3) $ED\ (kJ\ g^{-1}\ wm) = (1 - WF)\ [(LF_{wm} \times 39.9) + (PCF_{wm} \times 17.8)]$

(4) $ED\ (kJ\ g^{-1}\ dm) = (LF_{dm} \times 39.9) + (PCF_{dm} \times 17.8)$

2.2.4. Statistical analyses

Fatty acids were expressed as area percentage of total FA (%TFA) and plotted as mean ± standard error. A total of 59 and 56 FA were detected in 2013 and 2014 samples, respectively. Only those FA detected in both years were used for statistical analyses. Fatty acids in concentrations > 1.0 in each group (whale sharks, zooplankton and other invertebrates and small fish) were used for within-group comparisons. For among-group comparisons (potential prey groups and prey-predator comparisons), FA > 1.0 in whale shark profiles were used in analyses. Inter-annual analyses were only conducted with whale shark and zooplankton samples. The other prey collected in either 2013, 2014 or 2015 (e.g. larger zooplankton, cephalopods, krill and mysid) were pooled between years of sampling due to low sample size.

PERMANOVA (permutational multivariate analysis of variance, based on 9999 permutations) was used to test for factorial (collection time, sex and size class) differences in FA profiles and post-hoc pairwise comparisons were used to test for differences within levels or combinations of levels. Comparison of homogeneity dispersion between groups was performed using PERMDISP. Similarity percentage analysis (SIMPER) was used to identify the contribution of each FA to similarities or dissimilarities within and among groups. Non-parametric multi-dimensional scaling (MDS) was employed to visually explore relationships of groupings within and between whale sharks and potential prey items. Hierarchical cluster analysis based on group averages was applied in the MDS plot to show clustering of similar groups. All analyses

used PRIMER v6 software (Primer-E, UK) on untransformed data with a nonparametric Bray Curtis similarity matrix.

2.3. Results

2.3.1. Whale shark chemical composition

Whale shark sub-dermal tissue was low in lipid (4.0 ± 0.9 $mg.g^{-1}$ dry mass, dm) and high in water content (91.3 ± 2.6%) with a wet to dry mass ratio of 18.0 ± 5.2. Energy density for this tissue was estimated at 18.7 kJ g^{-1} dm (Table 2.1). Tissues collected in 2013 and 2014 were dominated by phospholipids (mean relative values of 72%), with minor lipid classes in order of decreasing importance including sterols, triacylglycerols, wax esters and free fatty acids (Table 2.1).

There was no significant difference in the FA composition between the outer and inner layers of sub-dermal tissue of whale sharks (PERMANOVA pseudo F = 0.519, p = 0.97). Both layers had similar percentages of polyunsaturated fatty acids (PUFA, 35.8% in the outer and 35.4% in the inner layer), followed by saturated fatty acids (SFA; 34.4%) in the outer layer and by monounsaturated fatty acids (MUFA, 30.4%) in the inner layer (Appendix 2.1).

Table 2.1. Biochemical data for whale shark sub-dermal tissue and zooplankton collected at Ningaloo Reef in May 2013 and May 2014.

Parameter	Unit		Whale sharks (n = 52)	Zooplankton (n = 56)
Water content	%		91.3 ± 2.6	- ± -
Wet/dry ratio	-		18.0 ± 5.1	- ± -
Lipid content	$mg.g^{-1}$	dm	4.0 ± 0.9	42.7 ± 2.6
	$mg.g^{-1}$	wm	0.4 ± 0.0	- ± -
Protein and carbohydrates	%	dm	95.0 ± 0.0	- ± -
Energy density	$KJ.g^{-1}$	dm	18.7 ± 0.2	- ± -
	$KJ.g^{-1}$	wm	0.1 ± 0.1	- ± -
Lipid class composition				
Wax esters	%		3.7 ± 1.3	6.5 ± 1.1
Triacylglycerols	%		7.7 ± 2.3	11.5 ± 1.6
Free fatty acids	%		2.0 ± 0.6	7.8 ± 1.4
Sterols	%		14.6 ± 1.3	5.2 ± 0.8
Phospholipids	%		71.9 ± 3.0	69.0 ± 3.2

Values are in dry mass and wet mass (dm and wm).
For comparative studies, 1 kilocalorie = 4.184 Kilojoules (kJ).

Table 2.2. The mean fatty acid (FA) composition (% of total FA ± standard error) of whale shark biopsies and zooplankton collected at Ningaloo Reef in May 2013 and May 2014.

Fatty acid	Whale shark				Zooplankton			
	2013 (n = 19)		2014 (n = 33)		2013 (n = 54)		2014 (n = 14)	
14:0	0.1 ± 0.1		0.2 ± 0.0		6.9 ± 0.5		3.3 ± 0.5	
i15:0	0.0 ± 0.0		0.1 ± 0.0		0.3 ± 0.0		0.2 ± 0.0	
15:0	0.1 ± 0.0		0.2 ± 0.0		1.4 ± 0.1		1.0 ± 0.1	
i16:0	0.1 ± 0.0		0.0 ± 0.0		0.6 ± 0.1		0.0 ± 0.0	
16:0	11.9 ± 1.4		9.7 ± 0.5		34.1 ± 1.7		19.4 ± 0.7	
i17:0	0.5 ± 0.1		1.2 ± 0.1		0.4 ± 0.0		0.3 ± 0.0	
17:0	0.8 ± 0.1		1.0 ± 0.0		2.4 ± 0.1		1.8 ± 0.1	
i18:0	0.5 ± 0.1		0.7 ± 0.0		0.4 ± 0.1		0.3 ± 0.0	
18:0	32.0 ± 3.4		18.0 ± 0.5		11.5 ± 0.8		7.6 ± 0.3	
20:0	0.8 ± 0.1		0.8 ± 0.4		1.1 ± 0.2		0.5 ± 0.0	
22:0	0.8 ± 0.2		0.5 ± 0.0		1.3 ± 0.3		0.5 ± 0.0	
24:0	0.8 ± 0.1		0.6 ± 0.1		0.7 ± 0.1		0.4 ± 0.1	
Total SFA	**48.5 ± 3.2**		**33.2 ± 0.8**		**61.3 ± 2.7**		**35.3 ± 1.0**	
16:1ω9c	0.2 ± 0.0		0.3 ± 0.0		0.3 ± 0.0		0.2 ± 0.0	
16:1ω7c	0.9 ± 0.3		1.3 ± 0.1		3.2 ± 0.3		3.3 ± 0.2	
16:1ω5c	0.0 ± 0.0		0.0 ± 0.0		0.3 ± 0.0		0.2 ± 0.0	
16:1ω13t	0.1 ± 0.0		0.2 ± 0.0		0.3 ± 0.0		0.1 ± 0.0	
17:1ω8c+a17:0	0.6 ± 0.1		1.1 ± 0.0		0.5 ± 0.1		0.3 ± 0.0	
17:1	1.7 ± 0.3		2.5 ± 0.2		0.1 ± 0.0		0.4 ± 0.1	
18:1ω9c	13.1 ± 1.9		15.6 ± 0.7		6.1 ± 0.6		5.5 ± 0.4	
18:1ω7c	3.5 ± 0.5		4.1 ± 0.4		1.9 ± 0.2		2.2 ± 0.2	
18:1ω7t	0.1 ± 0.0		0.2 ± 0.1		0.1 ± 0.0		0.1 ± 0.0	
20:1ω11c	0.4 ± 0.1		0.1 ± 0.0		0.3 ± 0.0		0.4 ± 0.1	
20:1ω9c	1.2 ± 0.4		1.4 ± 0.2		0.6 ± 0.1		0.6 ± 0.1	
20:1ω7c	0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.0	
20:1ω5c	0.7 ± 0.2		0.2 ± 0.0		0.0 ± 0.0		0.1 ± 0.0	
22:1ω11c	0.9 ± 0.6		0.5 ± 0.2		1.1 ± 0.3		0.2 ± 0.1	
22:1ω9c	0.7 ± 0.2		0.6 ± 0.3		0.2 ± 0.0		0.2 ± 0.0	
22:1ω7c	0.1 ± 0.0		0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.0	
24:1ω11c	0.0 ± 0.0		0.2 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
24:1ω9c	0.8 ± 0.1		1.6 ± 0.1		1.1 ± 0.1		1.2 ± 0.2	
Total MUFA	**25.4 ± 2.6**		**30.7 ± 1.2**		**16.9 ± 0.9**		**15.8 ± 0.7**	
18:3ω3	0.1 ± 0.0		0.6 ± 0.3		0.9 ± 0.3		1.2 ± 0.2	
18:4ω3	0.0 ± 0.0		0.3 ± 0.1		0.8 ± 0.1		1.6 ± 0.3	
18:3ω6	0.0 ± 0.0		0.1 ± 0.0		0.2 ± 0.0		0.5 ± 0.1	
18:2ω6	0.7 ± 0.0		1.0 ± 0.1		1.3 ± 0.2		1.6 ± 0.1	
20:4ω6 (ARA)	12.5 ± 1.7		16.4 ± 1.0		0.9 ± 0.1		2.7 ± 0.6	
20:5ω3 (EPA)	1.7 ± 0.2		2.0 ± 0.3		4.6 ± 0.6		10.2 ± 0.9	
20:3ω6	0.2 ± 0.0		0.3 ± 0.0		0.3 ± 0.1		0.2 ± 0.0	
20:4ω3	0.2 ± 0.0		0.6 ± 0.1		0.4 ± 0.0		0.5 ± 0.1	
20:2ω6	0.4 ± 0.2		0.2 ± 0.0		0.5 ± 0.1		0.4 ± 0.1	
21:5ω3	0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.2 ± 0.0	
22:5ω6	0.6 ± 0.1		1.0 ± 0.1		1.0 ± 0.1		1.3 ± 0.1	
22:6ω3 (DHA)	2.4 ± 0.2		3.5 ± 0.3		9.9 ± 1.2		27.2 ± 1.5	
22:4ω6	5.3 ± 0.7		6.8 ± 0.5		0.2 ± 0.0		0.4 ± 0.0	
22:5ω3	2.0 ± 0.2		3.2 ± 0.3		0.5 ± 0.1		0.9 ± 0.1	
Total PUFA	**26.1 ± 2.9**		**36.2 ± 0.9**		**21.7 ± 2.1**		**49.0 ± 1.3**	
ω3/ ω6	**0.3 ± 0.1**		**0.5 ± 0.1**		**3.7 ± 0.3**		**6.7 ± 0.6**	
Others	0.2 ± 0.0		0.2 ± 0.0		0.4 ± 0.0		0.2 ± 0.0	

Others (<0.2%): a15:0, 14:1ω7c, 16:1ω7t and 18:1ω5c.
SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid. t: trans-configured MUFA, c: cis-configured MUFA. The suffix i denotes branched fatty acids from the iso-series. FALD: fatty aldehyde analysed as dimethyl acetal.

Profiles of whale shark FA differed significantly between years (pseudo F = 12.057, p < 0.001). In 2013, profiles were largely dominated by SFA (48.5%), followed by PUFA (26.1%) and MUFA (25.4%). In contrast, 2014 biopsies were dominated by PUFA (36.2%) followed by SFA (33.2%) with slightly lower levels of MUFA (30.7%, Table 2.2). Heterogeneity in multivariate dispersion of FA was revealed between the two years (PERMDISP F = 13.62, p = 0.005) with 2013 samples showing lower within-group similarity (70.2%, SIMPER) than samples collected in 2014 (83.2%, SIMPER). Major FA for 2013 samples were 18:0, 18:1ω9, 20:4ω6 (ARA, arachidonic acid) and 16:0 and major FA for 2014 samples were 18:0, ARA, 18:1ω9 and 16:0 in decreasing order of relative abundance (Table 2.2). SIMPER analysis indicated that a slight increase of ARA and 18:1ω9 (1.5 fold) and a decrease of 18:0 (two-fold) between 2013 and 2014, contributed most to the separation between years. In both years, the mean ω3/ω6 PUFA ratios was < 1 and dominated by ARA (Table 2.2).

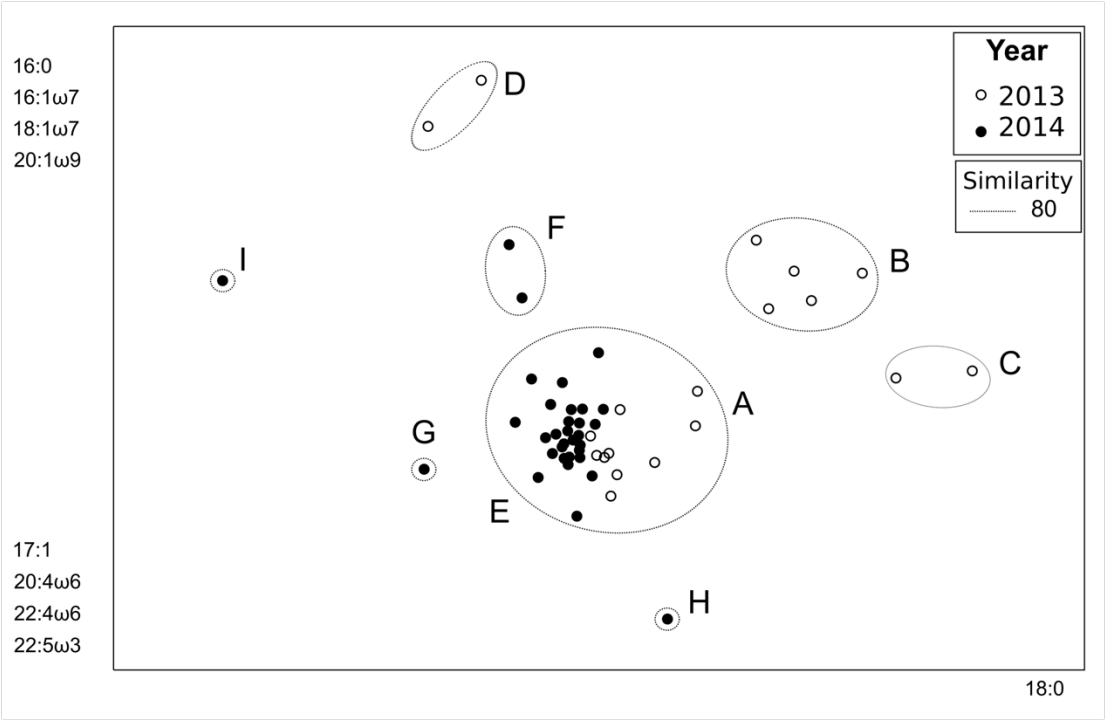

Figure 2.2. Multi-dimensional scaling ordinations of whale shark clusters (A, B, C, D, E, F, G, H and I) for 2013 and 2014. Fatty acids labeled represent the main coefficients (> 0.6) contributing to each axis.

Cluster analyses revealed distinct groups of whale shark FA profiles in both years: four in 2013 (A, B, C, D) and five in 2014 (E, F, G, H, I) (Fig. 2.2). Profiles of sharks in group A (2013) and group E (2014), which formed the central cluster of samples, were significantly different (t-test, $t = 2.947$, $p < 0.001$), although all were characterized by moderate levels of the four major FA (16:0, 18:0, 18:1ω9 and ARA) (SIMPER). The FAs that separated each group from the main cluster of samples in each year are summarized in Table 2.3. SIMPER analyses revealed that whale shark groups B and C (23.5%) and groups E and F (24.7%) were the most similar in 2013 and 2014, respectively. In contrast, the highest dissimilarities occurred between groups D and C (66.6%) and groups H and I (54.3%) in 2013 and 2014, respectively. Between years, groups C and I were the most dissimilar (63.1%, SIMPER; Fig. 2.2). No significant differences were detected in FA profiles of whale sharks according to sex (pseudo $F = 0.672$, $p = 0.707$) or size class (pseudo $F = 0.51$, $p = 0.875$).

Table 2.3. Collection and biological information of whale sharks analysed and grouped in this study by 80% similarity clusters of fatty acid profiles. Fatty acid indicators according to difference to main cluster groups: A in 2013 and E in 2014 (SIMPER).

Cluster	Sex	Size class (m)	Number	Fatty acid indicators
2013				
A (n = 10)	F	>6-8	1	Moderate levels of 18:0, ARA, 18:1ω9, 16:0
	M	<4	2	Slightly higher 18:0 than group E
		4-6	2	
		>8	1	
	U	4-6	3	
		>8	1	
B (n = 5)	M	4-6	3	Higher 16:0 and 18:0, lower ARA
	U	4-6	2	
C (n = 2)	M	<4	1	Much higher 18:0 and 16:0, lower ARA and 18:1ω9
		>6-8	1	
D (n = 2)	M	<4	2	Higher 18:1ω9 and 16:0, lower ARA
2014				
E (n = 28)	F	<4	1	Moderate levels of 18:0, ARA, 18:1ω9, 16:0
		4-6	6	Slightly higher 18:1ω9 than group A
	M	<4	1	
		4-6	15	
		>6-8	4	
	U	4-6	1	
F (n = 2)	M	4-6	1	Lower ARA and 18:0, higher 18:1ω9
		>6-8	1	
G (n = 1)	M	>6-8	1	Much lower ARA, higher 18:0
H (n = 1)	M	4-6	1	Higher 22:4ω6, lower 18:1ω9
I (n = 1)	F	>6-8	1	Lower ARA, 18:0 and 18:1ω9

F = female, M = male, U = unknown.
ARA: arachidonic acid.

2.3.2. Potential prey chemical composition

Zooplankton

Zooplankton samples were dominated by copepods, mainly from the genus *Paracalanus*, *Oncaea* and *Farranula* sp., followed by decapod larvae and foraminifers. The mean lipid content for all zooplankton samples was 42.7 ± 2.6 $mg.g^{-1}$ dm and was dominated by phospholipids followed by triacylglycerols, free fatty acids, wax esters and sterols (Table 2.1).

There were no significant differences in FA profiles of zooplankton sampled from different parts of the water column (surface, upper and bottom) in 2013 (pseudo F = 2.343, p = 0. 065) or of different size fractions (100, 300, 500 and 1000 μm) (pseudo F = 0.511, p = 0.904). Samples from different depths and size fractions were thus pooled for further analysis.

Overall, the FA composition of zooplankton differed significantly between years (pseudo F = 17.654, p < 0.001; Fig. 2.3A). FA profiles for zooplankton collected in 2013 showed lower within-group similarity (68%, SIMPER) and were dominated by SFA (61.3%) compared to the 2014 profiles that were PUFA-dominated (49%, Table 2.2). Major FA for 2013 samples were 16:0, 18:0, 22:6ω3 (DHA, docosahexaenoic acid), 18:1ω9 and 20:5ω3 (EPA, eicosapentaenoic acid) and major FA for the 2014 samples were DHA, 16:0, EPA, 18:0 and 18:1ω9, in decreasing order of abundance (Table 2.2). SIMPER analysis revealed that an increase of DHA (three-fold) and EPA (two-fold) and a decrease of 16:0 (two-fold) between 2013 and 2014, contributed most to the separation between years. Fatty acid profiles of larger zooplankton were dominated by SFA (50.4%, Appendix 2.2). SIMPER showed a higher influence of 18:0 and 18:1ω9 and a lower influence of DHA in these taxa than in overall zooplankton profiles (Appendix 2.2). The mean ω3/ω6 PUFA ratio, dominated by DHA, was consistently > 1 for all zooplankton and almost doubled in 2014 compared to 2013 (Table 2.2).

Other invertebrates and small fish

In 2013, we collected a wide range of small pelagic fish (*Spratelloides* sp, myctophids, reef fish juveniles (genera *Lethrinus*, *Chromis*, *Stegastes* sp., families *Mullidae* and *Synodontidae*)) cephalopods, annelids, crab larvae, decapods and isopods. In addition to these same taxa, krill (*Thysanopoda tricuspidata*), mysids and amphipods were collected in 2014. In 2015, an additional species of krill (*Pseudeuphausia latifrons*), was collected and included in the study.

The lipid content of all these organisms ranged from 9.7 (larger zooplankton) to 117.7 (cephalopods) $mg.g^{-1}$ dm (Appendix 2.3). While most groups were dominated by phospholipids, crab larvae and isopods also had high levels of triacylglycerols (Appendix 2.3).

The fatty acid profiles of most taxa were dominated by PUFA (32.4 - 56.4%), with the exception of pelagic and reef fish larvae and phytoplankton samples, which had higher contributions of SFA (40.7% - 49.7%) than the remaining taxa. Overall, most of the taxa including cephalopods, crab larvae, decapods, isopods and both species of krill were dominated by 16:0 and 18:0, whereas the PUFA fraction was composed of high levels of DHA, lower levels of EPA and low levels of ARA, grouping closer in the MDS plot (Fig. 2.3A). The MUFA 18:1ω9 also occurred at high levels with a mean value of 10% (Appendix 2.2). Fish were characterized by higher levels of 18:0 and lower levels of EPA and ARA, whereas mysids and amphipods showed higher contributions of ARA than any other taxa (Appendix 2.2). The FA profile of annelids was the most dissimilar to other prey taxa, with relatively high levels of 18:0 and EPA and lower levels of DHA (SIMPER; Fig. 2.3A, Appendix 2.2). Among all prey taxa, SIMPER analysis revealed high abundances of 18:1ω9, 18:0 and DHA in phytoplankton, whereas levels of EPA, ARA and 16:0 were higher in *Sargassum*. All prey taxa had ω3/ω6 PUFA ratios > 1 dominated by DHA, with the exception of the annelids and *Sargassum* sp. which were EPA-dominated (Appendix 2.2).

2.3.3. Potential prey-predator fatty acid comparisons

Fatty acid profiles of whale sharks sampled in 2013 and 2014 were significantly different to those of all potential prey we sampled (t-test; $t = 2.162 - 9.198$, $p < 0.019$; Fig. 2.3B). SIMPER revealed that high levels of 18:0, 18:1ω9 and ARA in whale sharks and 16:0, DHA and EPA in potential prey were the main cause of these differences. Profiles of whale shark groups C, H and G were the most different to those of prey taxa, whereas groups F, D and B clustered towards the center of the MDS plot closest to the large grouping of a variety of different prey types (SIMPER, dissimilarity 41% - 67%; Fig. 2.3B).

Despite differences between profiles of whale sharks and prey groups, both whale sharks and zooplankton displayed similar inter-annual trends, with SFA dominating profiles in 2013 and PUFA in 2014 (Table 2.2). High levels of 16:0 and 18:0 in annelids and high levels of ARA in mysids and amphipods, made these groups the most similar to whale sharks than any other prey category (SIMPER, dissimilarity 44% - 47%; Fig. 2.3B). In contrast, both species of krill and isopods clustered the furthest from whale sharks due to low levels of 18:0 and ARA and higher levels of DHA and EPA (SIMPER, dissimilarity 55%; Fig. 2.3B). Profiles of larger

zooplankton were more similar to whale sharks than overall zooplankton samples in both years, mainly due to higher levels of 18:0 and 18:1ω9 (SIMPER, dissimilarity 47%; Fig. 2.3B). Although a higher influence of ARA was found in *Sargassum* samples, phytoplankton was slightly more similar to whale sharks, mostly due to higher levels of 18:0 and 18:1ω9 (SIMPER, dissimilarity 46% - 49%, Fig. 2.3B).

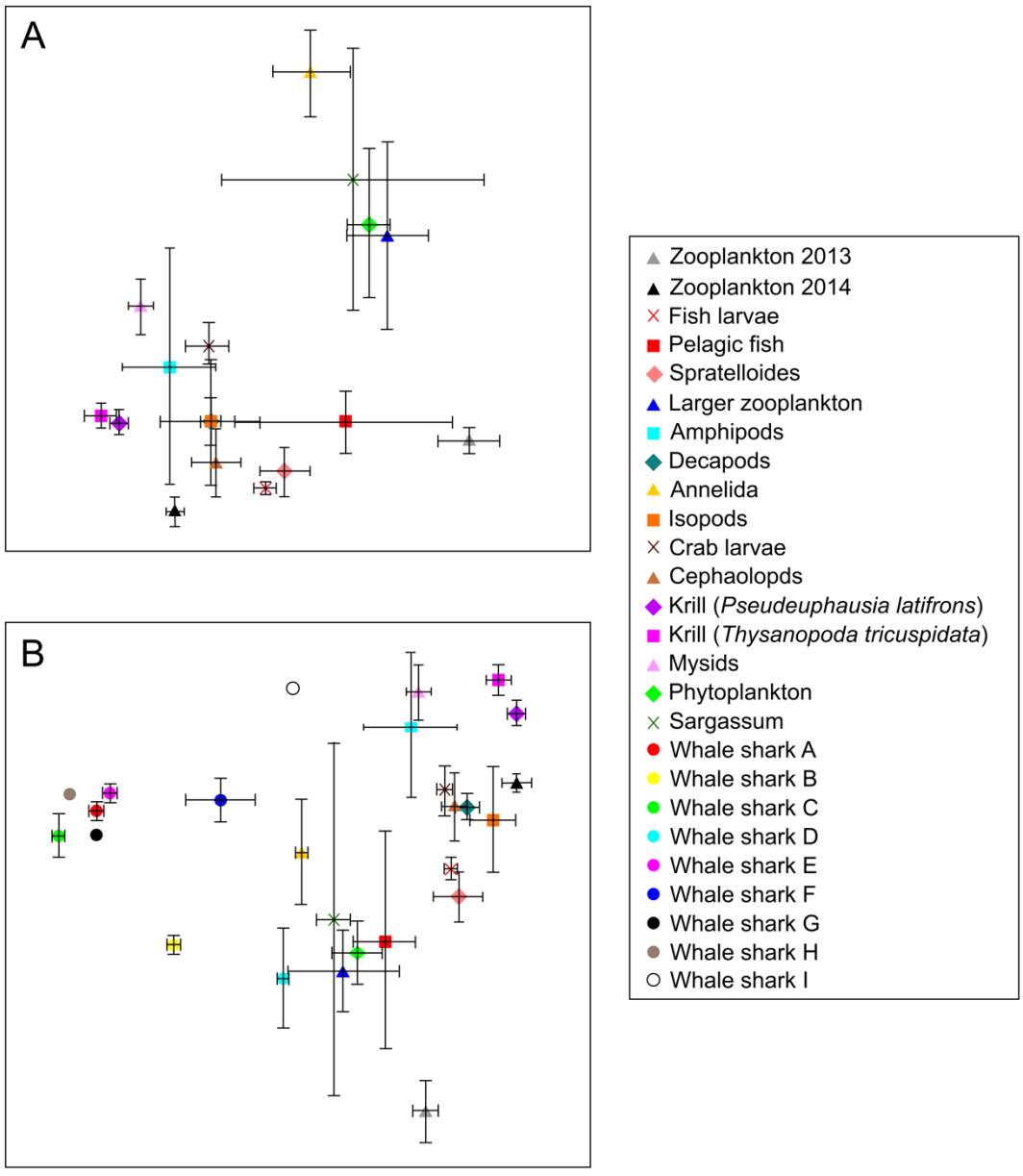

Figure 2.3. Multi-dimensional scaling of mean fatty acid profiles (% of total FA ± standard error) of (A) potential prey and (B) whale sharks and potential prey collected at Ningaloo Reef in May 2013, 2014 and 2015. Error bars represent standard error of different samples within each group.

2.4. Discussion

2.4.1. Chemical composition of whale shark sub-dermal tissue

The sub-dermal tissue of whale sharks collected at Ningaloo Reef was high in water (91.3%) and had chemical characteristics typical of a structural tissue with low lipid (4.0 $mg.g^{-1}$ dm or 0.4 $mg.g^{-1}$ wm) and energy content (18.7 $kJ.g^{-1}$ dm or 0.1 $kJ.g^{-1}$ wm), only low to moderate levels of TAG (energy storage lipid, 7.7% of total lipid) and high levels of PL (structural lipid, 72% of total lipid). Sub-dermal tissue had a much lower lipid content than that reported for the livers of other shark species (10 - 75% wm; Sargent et al. 1973, Nichols et al. 2001, Jayashinghe et al. 2003, Reme et al. 2006, Pethybridge et al. 2010, 2014, Davidson et al. 2014), a result that reflects the function of liver as an organ for lipid storage (Sheridan, 1988). Additionally, sub-dermal tissue also tended to be slightly lower in lipid content than muscle tissues reported in other elasmobranchs (0.2 - 3.6% wm; Sargent et al. 1973, Pethybridge et al. 2010, 2014, Couturier et al. 2013a, Davidson et al. 2014). Despite the superficial resemblance of the sub-dermal layer of whale sharks to the blubber layer of marine mammals (see Meekan et al. 2015), our results suggest that this tissue is unlikely to have a role in lipid storage.

The lipid content of sub-dermal tissue of whale sharks we sampled in the eastern Indian Ocean at Ningaloo Reef was lower (4.5 fold) than the values recorded for the same species in coastal waters off Mozambique in the western Indian Ocean (lipid content of 1.8 $mg.g^{-1}$ wm, Couturier et al. 2013b, Rohner et al. 2013). This variation in lipid content could reflect the different nutritional conditions (e.g. primary productivity) in each of these locations.

Although FA signatures of tissues, including sub-dermal tissue, liver and muscle, have been used as indicators of diet in both whale sharks and other elasmobranchs (Pethybridge et al. 2011, McMeans et al. 2013, Couturier et al. 2013 a,b, Rohner et al. 2013, Beckmann et al. 2014, Pethybridge et al. 2014), the interpretation of FA analyses presently remains hindered by our limited knowledge of lipid metabolism and fatty acid biosynthesis by elasmobranchs. For example, previous studies of deep-sea (squaliformes, chimaeriformes, hexanchiformes and carcharhiniformes) and Greenland sharks (*Somniosus microcephalus*) have demonstrated that dietary FAs are selectively incorporated into different tissues according to their metabolic and functional role (Pethybridge et al. 2010, McMeans et al. 2012). In addition, the time frame over which dietary FA are incorporated is likely to differ among tissues (Beckmann et al. 2014), since laboratory studies based on stable isotopes have shown that structural tissues such as muscle, fin and cartilage had slower turnover rates than more

metabolically active tissues such as liver or blood (e.g. Logan and Lutcavage, 2010, Hussey et al. 2011, Kim et al. 2012). However, the fibrous nature of the sub-dermal tissue coupled with poor vascularization (we recorded no signs of bleeding when biopsies were removed) suggests low metabolic activity and thus slower turnover rates of fatty acids than occurring in tissues such as muscle or blood. Profiles of sub-dermal tissues may thus reflect longer-term (up to several months) dietary signatures. Taking into account that biopsies were collected at the middle of the whale shark season, comparison of FA profiles from slower and faster turning over tissues will help elucidate whether sub-dermal tissue signatures are representative of feeding events while at Ningaloo or to prior arriving to the aggregation.

Several studies have reported vertical stratification of FA profiles within tissues (see review by Budge et al. 2006). For example, the blubber layer of marine mammals has marked stratification in FA composition with the more metabolically-active inner layer reflecting more recent diet than the outer layer closer to the skin surface (Best et al. 2003, Budge et al. 2008). In contrast, we found no stratification in total lipid or FA profiles in whale shark biopsies. This finding may indicate that all of the sub-dermal tissue has a slow rate of turnover, and hence further supporting the idea that this tissue provides a medium to longer-term indicator of diet.

2.4.2. Temporal and intraspecific variation in whale shark fatty acids

At Ningaloo Reef, there was high variability in the FA profiles of whale sharks both within and between years, with the MDS analysis identifying four different groups of whale sharks in 2013 and five groups in 2014. In other wide-ranging animals, such as seals, whales and seabirds, variability in FA profiles among individuals or groups of individuals has been linked to spatial variation in diet (e.g. Bradshaw et al. 2003, Iverson et al. 2007, Budge et al. 2008). Because these large, filter-feeding sharks face the challenge of meeting high energetic demands while consuming prey that are many orders of magnitude smaller than their own body size, similar to basking sharks (*Cetorhinus maximus*), whale sharks target dense aggregations of prey (Sims and Quayle, 1998, Rohner et al. 2015) in order to enhance cost-efficiency when foraging. As food is patchy both in space and time in oligotrophic tropical oceans, horizontal movements of whale sharks in search of prey are thus likely to show considerable variation among individuals. This idea is consistent with satellite tagging studies that show that after departing Ningaloo Reef, individual whale sharks travel north towards the equator using many different routes (Wilson et al. 2006, Sleeman et al. 2010). Similarly, Hueter et al. (2013) showed that individual whale sharks took different paths when travelling towards the Caribbean Sea and the South Atlantic after departing an aggregation off the Yucatan Peninsula in Mexico. Thus, the high variability in the FA profiles of whale sharks both within

and between years at Ningaloo may reflect variation in the prey consumed by individuals or groups of sharks, both when travelling to and from Ningaloo, and while resident at the aggregation.

Although changes in feeding habits according to sex or size class are well documented in elasmobranchs (Wetherbee and Cortes, 2004), neither of these factors accounted for the variability in FA analyses we observed. Similar to many other localities (e.g. Belize, Maldives and Mozambique; Rowat and Brooks, 2012), the Ningaloo Reef aggregation is comprised mostly of juvenile males (Meekan et al. 2006) and our sampling reflected this skewed composition of the population. The low sample sizes of adults and females probably reduced the ability of our study to discern differences in FA analyses based on size or sex, given that such differences have been recorded in localities where aggregations consist of a wider range of sizes and more balanced sex ratio, such as in the Gulf of California (Ketchum et al. 2013) and off the coast of India (Borrell et al. 2011a). Broadening the sampling of sizes and increasing sampling of females should be an aim of future sampling.

The inter-annual shift from SFA to PUFA in fatty acid profiles of whale sharks and zooplankton between 2013 and 2014 at Ningaloo Reef is likely a reflection of seasonal and/or inter-annual changes in the composition of primary producers. Oceanographic and environmental variations, such as changes in temperature, salinity, nutrients and light, can alter the FA composition of phytoplankton (Dalsgaard et al. 2003), which can in turn alter the composition of animals at higher trophic levels (Budge et al. 2002, 2008, Pethybridge et al. 2015). For example, a higher amount of unsaturation of FA, as we observed in 2014, could be due to the lower sea surface temperatures that occurred in this year (www.imos.org.au), since cooler waters are associated with an increase in the membrane fluidity of cells facilitated by unsaturated FA (Parrish 2013).

2.4.3. Food web inferences

Although observational studies have suggested that whale sharks feed on pelagic zooplankton (see review Rowat and Brooks, 2012), results of the FA profiles presented here and those of earlier studies of both whale sharks off the coast of Mozambique and manta rays (*Manta alfredi*) (Couturier et al. 2013 a,b, Rohner et al. 2013) suggest a wider foraging range for these tropical large filter-feeders. Rather than profiles dominated by the ω3 FA, DHA and EPA, which are typical of pelagic systems (Dalsgaard et al. 2003) and are also found in high concentrations in the potential prey collected in this study (Appendix 2.3), we found that FA profiles of the sub-dermal tissue of whale sharks were dominated by the ω6 FA, ARA.

Couturier et al. (2013 a,b) and Rohner et al. (2013) have recently suggested that the high ω6 LC-PUFA signatures observed in whale sharks and manta rays might be linked to deep water foraging that included demersal zooplankton such as mysids that emerge nocturnally from the sediment into the water column, and deeper living fish and macrozooplankton.

Fatty acid profiles that include high levels of ARA are characteristic of benthic or benthopelagic organisms, including echinoderms, amphipods, fish and demersal zooplankton (e.g. Copeman and Parrish, 2003, Connelly et al. 2014). High ARA levels are also found in some macroalgae (e.g. Virtue and Nichols, 1994, Johns et al. 1979). Although not fully understood, the trophic path of this ω6 PUFA in the marine environment might originate from microheterotrophs and protists such as thraustochytrids, which are ubiquitous in oceanic environments including sediment (Nichols et al. 2003, Lee Chang et al. 2012). Of the potential prey collected in this study, amphipods and mysids, which are known to be part of the demersal zooplankton in coastal habitats (Alldredge and King, 1985), and *Sargassum* sp. showed high levels of ARA. Although fragments of macroalgae and mysids have been reported in whale shark stomachs in Mozambique and South Africa (Rohner et al. 2013), the ingestion of the former by whale sharks is likely to only be incidental. Satellite tagging has revealed that whale sharks in the open ocean dive to meso- bathypelagic depths (200 - 1000 m) (Graham et al. 2006, Wilson et al. 2006, Brunnschwiler and Sims, 2011). These deep dives are thought to be foraging related (Meekan et al. 2015). In addition, stable isotope reports from India also indicated benthic foraging by whale sharks larger than 4 m, which showed enriched $\delta^{13}C$ values ranging from –17.4‰ to -15.1‰ (Borrell et al. 2011a), values associated to marine benthic algae (France 1995). Therefore, the link to high ARA in whale shark FA profiles is likely to come from direct ingestion of demersal zooplankton when close to the coast or other organisms in the deep scattering layer when off the continental shelf.

Although deep foraging is likely to account for FA profiles high in ARA, it is also possible that the higher relative levels of this FA is derived from other sources. For example, Wyatt et al. (2012b) found that fish in the Ningaloo area had profiles with ARA that they argued might be reef-derived from coralline algae and coral mucus (van Duyl et al. 2011). In addition, ARA in whale shark sub-dermal tissue could also have a metabolic origin. Whereas fishes are not able to biosynthesis LC-PUFA *de novo*, it is known that this group has metabolic pathways to elongate shorter chain (≤ C_{18}) PUFA to LC-PUFA (Monroig et al. 2013), an ability that could confound FA signatures derived from foraging. However, this idea is inconsistent with the high variability of ARA levels we found among individual sharks (SE, Table 2.2), suggesting that such differences are more likely to be related to diet rather than physiology. Our results

highlight the need to analyze tissues with faster rates of metabolic turnover, such as liver or blood, in order to differentiate between feeding and metabolic pathways for the accumulation of ARA in the sub-dermal tissues of whale sharks.

2.5. Conclusions

Our results are consistent with recent studies that suggest that whale sharks have a significant component of their diet that originates from benthic and deeper water habitats. Intraspecific variability in FA profiles implies differences in the diet of sharks aggregating at Ningaloo Reef that ultimately may reflect the patchy and unpredictable nature of foraging in oligotrophic tropical waters. Future studies need to target the sampling of tissues with higher rates of turnover, so that the relative inputs of past and recent feeding events on FA profiles can be assessed. Combining signature FA analysis with other techniques such as stable isotopes, genetic and long-term tagging data will also help better elucidate the feeding ecology and movements of this iconic species.

Chapter 3

Effects of sample treatment on the analysis of stable isotopes of carbon and nitrogen in zooplankton, micronekton and a filter-feeding shark

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Abstract

Stable isotope analysis is often used to investigate the trophic ecology of marine systems. However, a lack of standardization of the treatment of samples prior to analysis, hampers comparisons of results within and among studies. This study examined the effects of lipid extraction (LE), acidification for the removal of inorganic carbonate (RIC) and rinsing with deionized water (DIW) on δ^{13}C and δ^{15}N values and C:N ratios in sub-dermal tissue of whale sharks (*Rhincodon typus*), zooplankton (> 200 – 1000 µm) and a wide range of micronekton (> 1000 µm) taxa collected in 2013 and 2014 at Ningaloo Reef (Western Australia). For whale shark tissue, lipid extraction (LE and LE+DIW) increased values of δ^{13}C, whereas LE, LE+DIW and DIW treatments increased values of δ^{15}N and C:N ratios. These results confirm the need to remove lipids and ^{15}N-depleted nitrogenous waste from elasmobranch tissues. The LE+DIW treatment was the most efficient at achieving this goal. For zooplankton and micronekton, LE and RIC treatments had consistent effects on δ^{13}C values however, effects on δ^{15}N values were more unpredictable. Therefore, zooplankton and micronekton samples should be split into two portions, one subjected to LE or LE+RIC treatments to standardize δ^{13}C values, and a second untreated portion used for analysis of δ^{15}N values. For these taxa, the RIC+DIW treatment resulted in the greatest change in δ^{15}N values, which may confound results. Mathematical normalization models used to predict outcomes of treatments on values of δ^{13}C and δ^{15}N were not found to be suitable for all the taxa in this study.

3.1. Introduction

Stable isotope analyses (SIA) of carbon (δ^{13}C) and nitrogen (δ^{15}N) has allowed researchers to examine diet and food web interactions, track movements and investigate habitat use in a wide range of marine species (Fisk et al. 2002, Matich et al. 2011, Speed et al. 2012). Key to the use of SIA is the observation that enrichment in levels of δ^{13}C from prey to consumer can be used to determine carbon sources in food webs, whereas increases in levels of δ^{15}N provide information about trophic position (Peterson and Fry, 1987, Post 2002). Despite advances in understanding the limitations of the technique (e.g. trophic discrimination factors, isotopic incorporation and routing) (Martínez del Rio et al. 2009, Wolf et al. 2009), there are still contrasting results with regard to the effects of sample treatments on SIA. This hinders our ability to interpret results within or across studies. Standardized protocols for sample preparation are therefore needed for taxa across a range of trophic levels in order to use SIA to investigate diet composition and food web structures in ecosystems.

Lipid extraction of samples prior to analysis is common practice in SIA studies in order to standardize δ^{13}C values for comparison among tissues, individuals and species with variable lipid content (e.g. zooplankton, Syväranta and Rautio, 2010, Pomerleau et al. 2014, crustaceans, Bodin et al. 2007, teleosts, Sotiropoulos et al. 2004, Abrantes et al. 2011, marine mammals, Yurkowski et al. 2016, cephalopods, Ruiz-Cooley et al. 2011, elasmobranchs, Hussey et al. 2010, 2012). Removal of lipids is recommended since they are depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein, 1977), which can bias δ^{13}C values when compared across samples. Lipids are usually removed *a priori* by chemical extraction (e.g. chloroform:methanol or petroleum ether) (e.g. Hussey et al. 2012, Kim and Koch, 2012). However, a range of studies have shown that this can also affect δ^{15}N values, which might confound interpretation of data (Sotiropoulos et al. 2004, Murry et al. 2006, Sweeting et al. 2006, Ingram et al. 2007, Syväranta and Rautio, 2010). As changes in δ^{15}N values due to treatments are poorly understood, two sub-samples are often analysed; one lipid-extracted for estimating values of δ^{13}C and another untreated for estimating values of δ^{15}N. In order to avoid this double-handling of samples, mathematical normalization is sometimes used to correct for the presence of lipids (e.g. Kiljunen et al. 2006, Post et al. 2007, Logan et al. 2008). This approach can reduce analysis time and costs, but should be used with caution, as mathematical models are not necessarily applicable to all species (Abrantes et al. 2011).

The inorganic carbon present in many zooplankton, molluscs and fishes can also be a source of isotopic variability across samples (particularly in levels of δ^{13}C) and might confound

signatures of diet. Typically, the inorganic carbon in samples from these taxa is removed by acidification using HCl (Wada et al. 1991). However, recent studies present conflicting results on the effects of this treatment for δ^{13}C and δ^{15}N values. Although several studies have found no effect (Bosley and Wainright, 1999, Mitenbeck et al. 2008, Pomerleau et al. 2014), others showed variation in both δ^{13}C and δ^{15}N values after acidification (Bunn et al. 1995, Jacob et al. 2005, Carabel et al. 2006). Rinsing with deionized water after acidification is also a common method in SIA (e.g. Bunn et al. 1995, Jaschinski et al. 2008). However, the effects of this combined treatment on δ^{15}N values are still not fully understood. Consequently, there is still no consensus whether acidification and acidification followed by rinsing with deionized water is essential for SIA.

Examination of stable isotopes in elasmobranch tissues requires additional treatment steps since, unlike other marine organisms, these animals retain nitrogenous waste urea and trimethylamine oxide (TMAO) in their tissues for osmotic balance (Olson 1999). These nitrogenous compounds are depleted in ^{15}N, which may result in lower δ^{15}N values and an underestimation of trophic position (Fisk et al. 2002). Urea removal is therefore recommended by most studies, with a significant increase in δ^{15}N values found after lipid extraction and/or deionized water rinsing treatments of samples (Hussey et al. 2012, Kim and Koch, 2012, Churchill et al. 2015, Li et al. 2016, Carlisle et al. 2016, Burgess and Bennett, 2017). However, the outcome of these treatments can vary among species, since Logan and Lutcavage (2010) found no effect of urea on δ^{15}N values in skates (*Leucoraja* spp.) and spiny dogfish (*Squalus acanthias*). Mathematical models have been developed recently to correct isotope values for both lipid and urea in muscle tissue of different sharks (Churchill et al. 2015, Li et al. 2016) and may provide a means to avoid treatment of samples.

This study addresses these issues by investigating the effects of different treatments of samples on the analysis of stable isotopes of carbon and nitrogen in marine taxa from a variety of trophic levels. The outcomes of these treatments are then compared to those predicted by mathematical normalization models in order to assess the applicability of these models for the target taxa. Our objectives were to: i) assess the effects of lipid extraction and deionized water rinsing on δ^{13}C and δ^{15}N values and C:N ratios in whale shark (*Rhincodon typus* Smith, 1828) sub-dermal tissue; ii) assess the effects of lipid extraction, acidification and deionized water rinsing to δ^{13}C and δ^{15}N values and the C:N ratios in zooplankton (> 200 – 1000 µm) and a wide range of micronektonic taxa (> 1000 µm) including euphausiids, decapods, fish larvae, mysids, cephalopods and parasitic copepods; iii) develop mathematical correction models to investigate the relationship between lipid content, C:N ratio and δ^{13}C values using zooplankton

data; and iv) test existing lipid, urea and inorganic carbonate normalization models for use with whale shark sub-dermal tissue, zooplankton and micronekton taxa.

3.2. Materials and Methods

3.2.1. Sample collection

Samples of sub-dermal tissue of 15 whale sharks, 21 whale shark parasitic copepods, 315 pelagic invertebrates representing six taxa (zooplankton, euphausiids, decapods, fish larvae, mysids and cephalopods) (see Appendix 3.1 for a full list of samples), were collected at Ningaloo Reef, Western Australia (22° 33′ 45″ S, 113° 48′ 37″ E), during May 2013 and May 2014, coinciding with the annual aggregation of whale sharks at this location.

3.2.1.1. Whale shark sub-dermal tissue

Sub-dermal tissue was used because it is the largest proportion of tissue obtained in non-invasive biopsy sampling of large elasmobranchs. Sub-dermal tissue was obtained from biopsies of unrestrained wild whale sharks. Skin biopsies approximately two cm deep were collected using a modified hand spear fitted with a biopsy tip by snorkelers. A third of the biopsy, the inner part closer to the muscle, was immediately frozen (-20°C) and stored for SIA analysis.

3.2.1.2. Zooplankton and micronekton

Data derived from the parasitic copepods living on marine organisms can provide invaluable insights into the feeding ecology of their host (Deudero et al. 2002). Whale shark parasitic copepods (*Pandarus rhincodonicus)* were collected from the mouth and the body surface of whale sharks by a snorkeler and immediately frozen for further analysis. Zooplankton samples were collected using zooplankton nets (200 and 300 μm mesh) towed at the surface and at depth for 10 minutes at a speed of ~2 knots. Micronekton taxa that can avoid net capture including euphausiids, decapods, fish larvae, mysids and cephalopods (Wilson et al. 2003), were collected using two single-chamber light traps (see Meekan et al. 2001 for design). Traps were deployed overnight on moorings and suspended at the surface by buoys in the waters just off the reef crest. After collection, the following morning, all samples were transported to shore in insulated containers with ambient seawater, sorted to the lowest taxonomic group and frozen in separate cryovials (-20°C). All specimens were stored prior to SIA analysis and were analyzed whole.

3.2.2. Sample preparation

Samples were freeze-dried for 48 h and homogenized with a mortar and a pestle. Liquid nitrogen was added to tissue samples of whale sharks to reduce the tissue to a fine powder (Zybailov et al. 2005). Homogenized samples within each of the eight taxa were then pooled together. For whale shark sub-dermal tissue, five sub-samples (~5 mg) from the pool were assigned to each treatment: i) untreated (Control), ii) lipid extracted (LE), iii) deionized water rinsed (DIW) and iv) a combination of both techniques (LE+DIW) (Table 3.1). For zooplankton and micronekton taxa the same procedure as above was applied as follows: i) Control, ii) acidification to remove inorganic carbon (RIC) and iii) a combination of acidification and deionized water rinse (RIC+DIW). Due to low sample sizes and the ability of analytical method to detect small quantities, the following treatments were applied: iv) LE to all taxa but cephalopods, and v) a combination of lipid extracted and acidification (LE+RIC) to all taxa but mysids, cephalopods and parasitic copepods (Table 3.1).

Table 3.1. Summary of treatments on whale shark sub-dermal tissue, and zooplankton and micronekton taxa, + treatment applied, - treatment not applied.

Group	**Treatments**						
	Control	**LE**	**DIW**	**RIC**	**LE+DIW**	**LE+RIC**	**RIC+DIW**
Whale shark	+	+	+	-	+	-	-
Zooplankton	+	+	-	+	-	+	+
Euphausiids	+	+	-	+	-	+	+
Decapods	+	+	-	+	-	+	+
Fish larvae	+	+	-	+	-	+	+
Mysids	+	+	-	+	-	-	-
Cephalopods	+	-	-	+	-	-	+
Parasitic copepods	+	+	-	+	-	-	+

LE = lipid extraction; DIW = deionized water rinsing; RIC = acidification

To extract lipids (LE), sub-samples were left overnight in a methanol:chloroform:Milli-Q water (2:1:0.8 v/v/v) solution following a modified Bligh and Dyer (1959, Marcus et al. 2016) method and after breaking phase and removal of the lipid-containing layer, the aqueous-methanol layer was then vacuum filtered. The resulting lipid-free tissue was re-dried in the oven at 60°C for 48 h to remove the remaining solvent. Sub-samples that underwent DIW treatment were soaked in deionized water in 1.5 ml Eppendorf tubes for 24 h at room temperature. Tubes were then centrifuged for three minutes and water extracted using a needle syringe. This procedure was repeated three times and samples were then left to re-dry in the oven at 60°C. For the RIC treatment, sub-samples were fumigated with 2N HCl (King et al. 1998) in a

desiccator under vacuum for 24 h and then re-dried in the oven at 60°C. Combined treatments (LE+RIC and RIC+DIW) followed the above protocols consecutively.

3.2.3. Isotopic analysis

Between 0.4 to 0.7 mg of treated samples were weighed in tin capsules and carbon and nitrogen stable isotopes analyzed using flash combustion isotope ratio mass spectrometry (varioPYRO cube coupled to Isoprime100 mass spectrometer) at the Central Science Laboratory, University of Tasmania (Australia). Stable isotope abundances are reported in delta (δ) values as the deviations from conventional standards in parts per thousand (‰) from the following equation:

$$(1) \qquad \delta X = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right] \times 1000$$

where X = ^{13}C or ^{15}N and R = the ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The standard reference materials were Pee Dee Belemnite (PDB) for carbon and atmospheric nitrogen for nitrogen. International reference standards with known isotopic composition were measured for instrument calibration after every 6th sample. Stability of the instrumentation, analytical precision, drift correction and linearity performance were calculated from the repetitive analysis of these standards. Precision was ± 0.1‰ for both isotopes.

3.2.4. Urea concentration in different treatments

To determine and quantify urea removal in the LE and DIW treatments, three samples of the residual water after each treatment were analyzed for urea concentration with a Nuclear Magnetic Resonance spectrometer (NMR). The NMR spectra were acquired using a Bruker Avance III HD 600.07 MHz spectrometer with 5 mm TCI cryoprobe. Sample temperature was regulated at 298^{o}K. The Bruker pulse program “noesygppr1d” was configured with 64K data points, relaxation delay of 4 s, sweep width of 20 ppm and 32 transients. Residual water signal was attenuated by presaturation in the relaxation delay. Data were processed with an exponential multiplication apodisation with a line-broadening factor of 0.3 Hz. Measurements were made at a range of relaxation delay lengths to ensure values were appropriate relative to the T1 rates of the observed species.

Samples were dried and reconstituted in deuterated dimethylsulphoxide (d6-DMSO 99.9% Cambridge Isotope Laboratories, Andover MA, USA). A stock solution of trimethylsilylpropane sulphonic acid (TMSP) was made in d6-DMSO to serve as both chemical shift reference and quantitation standard.

3.2.5. Statistical analysis

Normality of data was assessed using Shapiro-Wilk normality tests and homogeneity of the variance using the Bartlett's test. A One-way ANOVA or a Generalized Least Square model (GLS) followed by a Tukey's multiple comparison were used to assess differences in $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios among treatments of each of the taxa. A GLS was used in those cases when there was no homogeneity of the variance. All statistical analyses used R v.3.0.1 software (Ihaka and Gentleman, 1996; http://www.r-project.org) with a criterion of $p < 0.05$.

3.2.6. Lipid effects on zooplankton isotopic signatures

A lipid normalization equation was generated based on the zooplankton data to investigate relationships between $\delta^{13}C$ values, lipid content and C:N ratios. For that purpose, a total of 15 different zooplankton samples were lipid extracted only, using the same method as above (Bligh and Dyer, 1959). These samples were randomly selected from different net tows undertaken at different stations at Ningaloo Reef in 2013. Lipid content (% of dry mass), the $\delta^{13}C$ values and the C:N ratio before and after lipid extraction were measured for each sample. The relationships between: i) lipid content and C:N ratio, ii) C:N ratio and $\Delta\delta^{13}C$ ($\Delta\delta^{13}C = \delta^{13}C_{LE} - \delta^{13}C_{bulk}$), iii) $\Delta\delta^{13}C$ and lipid content and iv) $\delta^{13}C_{LE}$ and $\delta^{13}C_{bulk}$ using simple linear regressions were then evaluated.

3.2.7. Mathematical normalization

Several published linear and non-linear models for mathematical normalization of $\delta^{13}C$ and $\delta^{15}N$ values were applied to the data. First, model correction from two different studies (Churchill et al. 2015, Li et al. 2016) were applied to data from whale shark sub-dermal tissue. Churchill et al. (2015) developed species-specific normalization models to predict lipid extracted values of $\delta^{15}N$ for three species of deep-sea sharks:

(2) *Centrophorus cf. granulosos* $\delta^{15}N_{LE} = 1.80 + 0.91 \times \delta^{15}N$

(3) *Squalus cubensis* $\delta^{15}N_{LE} = 3.47 + 0.77 \times \delta^{15}N$

(4) *Squalus cf. mistukurii* $\delta^{15}N_{LE} = 7.67 + 0.42 \times \delta^{15}N$

Normalization models by Li et al. (2016) were derived from a combination of data from muscle tissue of three pelagic sharks: silky (*Carcharhinus falciformis*), blue (*Prionace glauca*) and smooth hammerhead (*Shyrna zygaena*). The following equations used measured $\delta^{13}C_{LE}$ and $\delta^{15}N_{LE}$ values to calculate $\delta^{13}C_{LE+DIW}$ and $\delta^{15}N_{LE+DIW}$:

(5) $\delta^{13}C_{LE+DIW} = 0.954 \times \delta^{13}C_{LE} - 0.615$

(6) $\delta^{15}N_{LE+DIW} = 0.856 \times \delta^{15}N_{LE} + 2.813$

The selected lipid normalization equations used C:N ratios and/or $\delta^{13}C$ values of whole body bulk sample material to calculate lipid extracted $\delta^{13}C$ values ($\delta^{13}C_{LE}$) for zooplankton and micronekton. The models were developed from a wide variety of aquatic taxa with C:N ratios ranging from 2.9 – 19.3. The first of these was published by McConnaughey and McRoy (1979):

(7) $L = 93 / [1 + (0.246 \times C:N - 0.775)^{-1}]$

(8) $\delta^{13}C_{LE} = \delta^{13}C + D [I + 3.9 / (1 + 287 / L)]$

where *L* was lipid content, *D* was 6‰, the difference in isotopic composition between protein and lipid, and *I* was a constant value of -0.207. Kiljunen et al. (2006) revised the *D* and *I* parameters and estimated new values of 7.018 and 0.048, respectively. The second mathematical correction by Post et al. (2007), was derived from a wide range of aquatic animals:

(9) $\delta^{13}C_{LE} = \delta^{13}C - 3.32 + 0.99 \times C:N$

Logan et al. (2008), assumed a log-transformed relationship between C:N ratios and differences between $\delta^{13}C$ and $\delta^{13}C_{LE}$:

(10) $\delta^{13}C_{LE} = \delta^{13}C + \beta_0 + \beta_1 \ln (C:N)$

where β_0 and β_1 parameters were estimated in Logan et al. (2008) for different animal taxa. The final model was developed by Syväranta and Rautio (2010) for freshwater zooplankton:

(11) $\delta^{13}C_{LE} = \delta^{13}C + 7.95 \times [(C:N - 3.8) / C:N]$

The model by Pomerleau et al. 2014, which was developed for the shelled mollusc *Limacina helicina* to correct for inorganic carbonate was also applied to zooplankton and micronekton data:

(12) $\delta^{13}C_{A} = 0.994 \times \delta^{13}C - 1.096$

Student's t-tests were used to compare measured values of $\delta^{13}C$ and $\delta^{15}N$ after various treatments with those predicted by these models.

3.3. Results

3.3.1. Whale shark sub-dermal tissue

The mean (and standard error) of $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios of whale shark sub-dermal tissue after each treatment are shown in Table 3.2. There was a significant enrichment in ^{13}C following LE and LE+DIW. Mean $\delta^{13}C$ values increased by 1.7‰ for LE and 1.3‰ for LE+DIW treatments (Table 3.2, Fig. 3.1). However, there were no significant differences in $\delta^{13}C$ values between LE and LE+DIW (Fig. 3.1). In contrast, DIW treatment resulted in a significant depletion in ^{13}C with mean $\delta^{13}C$ values declining by 1.1‰ (Table 3.2, Fig. 3.1).

There was significant enrichment of ^{15}N following LE, DIW and LE+DIW treatments. The largest increase in mean $\delta^{15}N$ values followed DIW treatment (1.7‰), whereas LE yielded the smallest difference (0.9‰) (Table 3.2, Fig. 3.1). Urea concentrations found in the residual extracting water were on average 46.5 times higher after DIW than LE treatments (LE, mean = 110.4 ± 7.1 uM; DIW, mean = 5138.3 ± 931.7 uM). Values of $\delta^{15}N$ differed significantly between LE and DIW treatments (Fig 3.1). The effects of LE+DIW on $\delta^{15}N$ values were, however, not significantly different to those of LE or DIW treatments (Fig. 3.1).

The mean C:N ratio significantly increased following the LE, DIW and LE+DIW treatments by 1.5, 1.8 and 1.3, respectively (Table 3.2, Fig. 3.1), showing that proportionally, there was a greater removal of urea than lipids. The shift in the C:N ratio following DIW was significantly different to that observed after LE and LE+DIW (Fig. 3.1).

Table 3.2. Whale shark sub-dermal tissue $\delta^{13}C$ and $\delta^{15}N$ values (mean ± standard error ‰) and C:N ratios under different treatments: untreated (Control), lipid extracted (LE), deionized water rinsing (DIW), lipid extraction combined with deionized water rinsing (LE+DIW).

Parameter	Treatments			
	Control	LE	DIW	LE+DIW
$\delta^{13}C$	-17.1 ± 0.1	-15.4 ± 0.1	-18.2 ± 0.4	-15.8 ± 0.3
$\delta^{15}N$	8.1 ± 0	9 ± 0.1	9.8 ± 0.1	9.4 ± 0.2
C:N	1.6 ± 0	2.8 ± 0	3.4 ± 0.1	2.9 ± 0

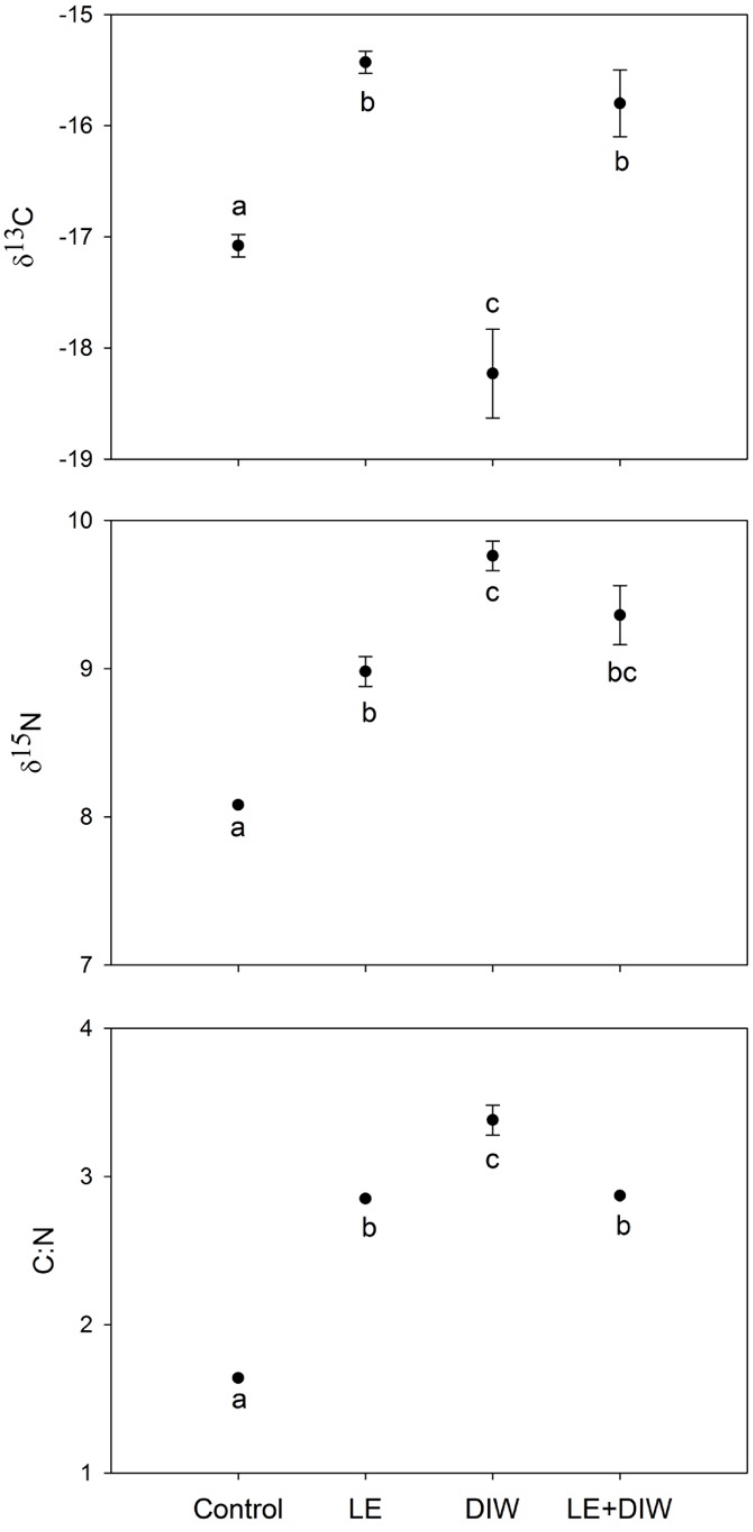


Figure 3.1. The effect on $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios among different treatments: untreated (Control), lipid extracted (LE), deionized water rinsing (DIW) and lipid extraction combined with deionized water rinsing (LE+DIW) of whale shark sub-dermal tissue. Treatments with different letters are significantly different ($p < 0.05$)

3.3.2. Zooplankton and micronekton

The effects of LE and RIC on the δ^{13}C and δ^{15}N values and C:N ratios were taxa-specific (Table 3.3, Appendix 3.2 and 3.3). Lipid extraction of samples (LE and LE+RIC) caused a significant enrichment in ^{13}C in all taxa examined, with the exception of zooplankton after LE+RIC. On average, the mean δ^{13}C values increased 0.7 ± 0.2‰ and 0.6 ± 0.2‰ following LE and LE+RIC, respectively, except in the case of zooplankton, which decreased by 0.3‰ after LE+RIC. The effects of LE and LE+RIC on δ^{13}C values differed significantly in all taxa, with the exception of fish larvae (Table 3.3). Significant differences in δ^{15}N values between LE and LE+RIC treated samples were only observed in decapod taxa. After LE and LE+RIC, zooplankton, decapods and parasitic copepods showed a significant depletion in ^{15}N, with mean δ^{15}N values decreasing up to 0.3‰. In contrast, euphausiids, fish larvae and mysids showed a significant increase in levels of δ^{15}N, with mean values increasing up to 0.8‰ (Table 3.3). The mean C:N ratio significantly decreased in all treated taxa by an average of 0.5 ± 0.1 ‰ and 0.7 ± 0.2 ‰, following LE and LE+RIC, respectively. However, only the C:N ratios of zooplankton and decapods differed significantly between the two treatments (Table 3.3).

There was a significant depletion in ^{13}C following RIC in samples from five of the seven taxa examined by this study. The largest decrease in the mean δ^{13}C values was found in decapods (0.6‰), whereas parasitic copepods showed the smallest shift of 0.1‰ (Table 3.3). Similarly, the mean C:N ratio of all taxa decreased (0.1 – 0.4‰) after acidification, but for cephalopods the ratio increased by 0.1‰. These shifts were significant only for zooplankton, mysids and parasitic copepods. Changes in δ^{15}N values were more variable among taxa. Significant shifts in values of δ^{15}N were only observed in zooplankton (0.6‰) and in cephalopods (0.2‰) (Table 3.3). Significant changes in δ^{13}C and δ^{15}N values and C:N ratios were found between RIC and all other treatments (LE, LE+DIW and RIC+DIW) for all taxa, with the exceptions of δ^{15}N values in decapods and in C:N ratios in euphausiids (Table 3.3).

Rinsing of samples with deionized water after acidification (RIC+DIW) resulted in a significant depletion in ^{13}C in all taxa. Maximum and minimum decreases in mean δ^{13}C values were found in decapods (2.1‰) and euphausiids (0.2‰), respectively (Table 3.3). In contrast, following RIC+DIW treatments, all taxa showed a significant increase in mean C:N ratios, although no significant change was detected for euphausiids. Cephalopods yielded the largest increase (1.8) and parasitic copepods the smallest (0.1) in ratios (Table 3.3). A significant depletion in ^{15}N followed RIC+DIW treatments of zooplankton, decapods and parasitic copepods, with mean δ^{15}N values declining by up to 1.1‰. In contrast, the same treatments of euphausiids

and fish larvae resulted in a significant enrichment of ^{15}N with mean $\delta^{15}N$ values increasing up to 0.7‰ (Table 3.3).

Table 3.3. Zooplankton and micronekton taxa $\delta^{13}C$ and $\delta^{15}N$ values (mean ± standard error ‰) and C:N ratios under different treatments: untreated (Control), lipid extracted (LE), acidification (RIC), lipid extraction combined with acidification (LE+RIC), acidification combined with deionized water rinsing (RIC+DIW). Treatment levels with different upper case letters are significantly different ($p < 0.05$) according One-way ANOVA test or GLS model.

		Variables											
Taxa	**Treatment**	**$\delta^{13}C$**				**$\delta^{15}N$**				**C:N**			
Zooplankton	Control	-19.5	±	0.1	A	+5.2	±	0.0	A	+4.3	±	0.0	A
	LE	-19.0	±	0.2	B	+4.9	±	0.0	B	+4.0	±	0.0	B
	RIC	-20.0	±	0.1	C	+5.8	±	0.1	C	+4.2	±	0.0	C
	LE+RIC	-19.8	±	0.0	A	+5.0	±	0.1	B	+3.7	±	0.0	D
	RIC+DIW	-20.5	±	0.1	D	+4.7	±	0.1	C	+4.7	±	0.0	E
Euphausiids	Control	-19.0	±	0.0	A	+6.7	±	0.0	A	+3.6	±	0.0	A
	LE	-18.2	±	0.1	B	+7.2	±	0.0	B	+3.2	±	0.0	B
	RIC	-19.0	±	0.0	A	+6.7	±	0.1	A	+3.2	±	0.2	ABC
	LE+RIC	-18.4	±	0.0	C	+7.2	±	0.0	B	+3.2	±	0.0	BC
	RIC+DIW	-19.2	±	0.0	D	+7.4	±	0.0	C	+3.6	±	0.0	A
Decapods	Control	-18.4	±	0.1	A	+6.7	±	0.0	A	+5.1	±	0.0	A
	LE	-17.3	±	0.1	B	+6.4	±	0.0	BC	+4.2	±	0.0	B
	RIC	-19.0	±	0.1	C	+6.6	±	0.1	AC	+5.0	±	0.1	A
	LE+RIC	-18.2	±	0.0	D	+6.6	±	0.0	A	+3.9	±	0.0	C
	RIC+DIW	-20.5	±	0.1	E	+5.6	±	0.2	D	+6.4	±	0.1	D
Fish larvae	Control	-18.5	±	0.1	A	+7.6	±	0.0	A	+3.6	±	0.0	A
	LE	-17.5	±	0.0	B	+8.4	±	0.0	B	+3.1	±	0.0	B
	RIC	-18.8	±	0.0	C	+7.8	±	0.0	A	+3.5	±	0.0	A
	LE+RIC	-17.5	±	0.0	B	+8.3	±	0.0	B	+3.1	±	0.0	B
	RIC+DIW	-19.2	±	0.1	D	+8.1	±	0.0	C	+4.0	±	0.1	C
Mysids	Control	-17.5	±	0.0	A	+8.5	±	0.0	A	+3.6	±	0.0	A
	LE	-17.0	±	0.0	B	+8.8	±	0.0	B	+3.2	±	0.0	B
	RIC	-17.7	±	0.0	C	+8.6	±	0.1	A	+3.5	±	0.0	C
Cephalopods	Control	-19.8	±	0.0	A	+8.9	±	0.0	A	+4.4	±	0.0	A
	RIC	-20.2	±	0.3	A	+8.7	±	0.0	B	+4.5	±	0.2	A
	RIC+DIW	-21.2	±	0.0	B	+9.0	±	0.1	A	+6.2	±	0.1	B
Parasitic copepods	Control	-16.8	±	0.0	A	+9.5	±	0.0	A	+4.0	±	0.0	A
	LE	-16.7	±	0.0	B	+9.3	±	0.0	B	+3.6	±	0.0	B
	RIC	-16.9	±	0.0	C	+9.6	±	0.0	A	+3.9	±	0.0	C
	RIC+DIW	-17.4	±	0.0	D	+9.2	±	0.0	B	+4.2	±	0.0	D

3.3.3. Lipid effects on zooplankton isotopic signatures

There was a significant positive relationship between $\Delta\delta^{13}C$ and lipid content in zooplankton samples. Percent lipid content explained 45% of the variation in $\Delta\delta^{13}C$ (Table 3.4). A significant positive relationship was also found between $\delta^{13}C_{LE}$ and $\delta^{13}C_{bulk}$ values, which explained 30% of the variation in $\delta^{13}C_{LE}$ values (Table 3.4). No significant relationship was found between C:N either with lipid content or with $\Delta\delta^{13}C$ (Table 3.4).

Table 3.4. Linear regression equations and diagnostic statistics used for examination of the effect of lipids on stable isotope analyses of zooplankton samples. Bold values indicate a significant relationship ($p < 0.05$).

Equations	n	Variance explained (R^2)	Significance (p-values)
(13) %lipid = 10.30 - 0.55 x C:N	15	0.001	0.879
(14) $\Delta\delta^{13}C$ = 2.96 - 0.60 x C:N	15	0.069	0.345
(15) $\Delta\delta^{13}C$ = -0.45 + 0.12 x %lipid	15	0.453	**0.005**
(16) $\delta^{13}C_{LE}$ = -12.44 + 0.33 x $\delta^{13}C$	15	0.308	**0.032**

3.3.4. Mathematical normalization

Predicted $\delta^{15}N_{LE}$ values calculated using the model provided by Churchill et al. (2015) for the deep-water shark *Centrophorus cf. granulosos* were not significantly different to those calculated for whale shark tissue, (t-test, t24= -2.25, p = 0.05). Values of $\delta^{13}C_{LE+DIW}$ predicted by the model provided by Li et al. (2016) did not differ from those observed by this study (t-test, t24= -1.53, p = 0.19). However, predicted values of $\delta^{15}N_{LE+DIW}$ were significantly greater (average difference 1.5 ± 0.0‰; t-test, t24= -5.09, p = 0.01) than those observed by the present study.

The models for lipid correction by McConnaughey and McRoy (1979), Post et al. (2007), Logan et al. (2008) and Syväranta and Rautio (2010) produced predicted values of $\delta^{13}C_{LE}$ that were significantly different to those measured for most taxa (t-test, t24= -37.40 – 32,31, p < 0.001 – 0.05). Exceptions were predictions for fish from models of McConnaughey and McRoy (1979) and decapods from models of Logan et al. (2008), which did not differ from observed $\delta^{13}C_{LE}$ values (t-test, t24= -1.25 - 0.63, p = 0.25 – 0.54). Predictions of the model by McConnaughey and McRoy (1979) tended to consistently overestimate $\delta^{13}C_{LE}$ values by up to 1.8‰ for most taxa. Similarly, values of $\delta^{13}C_{LE}$ predicted by the Post et al. (2007), Logan et al. (2008) and Syväranta and Rautio (2010) models were also overestimated (by 0.2 – 1.0‰)

compared to measured values for zooplankton, decapods and parasitic copepods. In contrast, predicted values for euphausiids, decapods and mysids were underestimated (by 0.2 – 1.6‰) compared to treatment results for these taxa. Correction for inorganic carbonate following the protocol of Pomerleau et al. 2014, produced predicted values of $\delta^{13}C_{RIC}$ that were significantly different than those observed in this study for all taxa with the exceptions of zooplankton and decapods (t-test, t24= -0.25 – 2.49, p > 0.05).

3.4. Discussion

3.4.1. Whale shark sub-dermal tissue

The significant increase in $\delta^{13}C$ values following chloroform:methanol extraction (LE and LE+DIW, 1.7‰ and 1.3‰, respectively) indicated removal of ^{13}C-depleted lipids from sub-dermal tissue, despite its low lipid content (~4.0 $mg.g^{-1}$ dry mass; Marcus et al. 2016). An increase in $\delta^{13}C$ values (up to 2.0‰) after lipid extraction was also reported in muscle of large sand tiger sharks (*Carcharias taurus*) under semi-controlled conditions and in other pelagic and benthic elasmobranchs (Hussey et al. 2010, 2012, Li et al. 2016, Carlisle et al. 2016, Burgess and Bennett, 2017). Hussey et al. (2012) reported a much higher change of 3.3‰ in muscle of whale shark tissue after LE. This marked difference in the shift of $\delta^{13}C$ values between sub-dermal and muscle tissue of whale sharks may be due to variable lipid content and possibly associated with the different functional roles of the tissue types (Marcus et al. 2016) and intraspecific differences in life-history and physiological condition of individuals (Pethybridge et al. 2010). Shifts in $\delta^{13}C$ values greater than 0.4‰, the estimated amount of enrichment between trophic levels (Post 2002), could lead to misinterpretations of diet source and movement patterns of these predators. As also noted by Carlisle et al. (2016), our study suggests that lipids need to be removed from elasmobranch tissues prior to analysis, even from those considered relatively lipid-poor, in order to standardize $\delta^{13}C$ values among tissues, individuals and species.

All treatments (LE, DIW and LE+DIW) resulted in the removal of isotopically light nitrogenous compounds (e.g. urea and TMAO) from sub-dermal tissue of whale sharks as indicated by both an increase in $\delta^{15}N$ values and C:N ratios. Following LE and LE+DIW, the increase in C:N ratios showed that there was a proportionally higher removal of urea and TMAO than lipid components, given the low lipid content of the sub-dermal tissue of whale sharks. These shifts in $\delta^{15}N$ values are within the very upper range (0.2 - 1.7‰) of those reported for muscle of whale sharks and for a wide range of deep water and pelagic species after similar treatment

(Hussey et al. 2010, 2012, Kim and Koch, 2012, Churchill et al. 2015, Li et al. 2016, Carlisle et al. 2016, Burgess and Bennett, 2017). However, a shift in δ^{15}N values of 1.7‰, and the abundant concentrations of urea found in the water rinses, showed that DIW was more effective at removal of nitrogenous waste than LE. The decrease in δ^{13}C values following DIW was expected, since urea is enriched in ^{13}C relative to dietary carbon (Ivlev et al. 1996, Kim and Koch, 2012). Whereas DIW does not alter protein composition (e.g. amino acids) of elasmobranch tissues (Kim and Koch, 2012), extraction by chloroform:methanol could have incidentally removed some proteins linked to structural lipids in membranes (e.g. lipoproteins), thus further altering δ^{15}N values, as has been suggested for fish and crustacean tissues (Sotiropoulus et al. 2004, Sweeting et al. 2006, Bodin et al. 2007). The effects of lipid extraction solvents on amino acid composition in elasmobranch tissues are a subject for future research.

Given urea and TMAO are ^{15}N-depleted, the failure to remove nitrogenous waste could represent a source of error when estimating the trophic position of an animal in a food chain. A diet-tissue discrimination factor of 5.1‰ for zooplanktivores (Hussey et al. 2014) suggests that if the δ^{15}N values were not adjusted by removal of urea and TMAO, the position of whale sharks would be under-estimated by ~0.3 of a trophic level. This bias could become greater for higher order consumers, as isotopic discrimination narrows in upper trophic levels (Hussey et al. 2014). For these reasons, and the intra- and inter-specific variability in urea concentrations in elasmobranchs due to physiological and environmental factors (Hussey et al. 2012), the need to remove urea and TMAO from elasmobranch tissues prior to analysis is clear, in order to avoid their presence significantly altering δ^{15}N values and resulting in misleading reconstructions of food webs.

The LE+DIW treatment was the most efficient for removing both lipids and urea in this study, similar to the recent findings of Kim and Koch (2012), Carlisle et al. (2016) and Li et al. (2016). As the outcome of the LE+DIW treatment did not differ from individual applications of each component (Fig. 3.1), it is recommended as a means to standardize δ^{13}C and δ^{15}N values in elasmobranch tissues prior to SIA.

3.4.2. Zooplankton and micronekton

Increases in δ^{13}C values after lipid extraction have been reported by many studies (e.g. McConnaughey and McRoy, 1979, Murry et al. 2006, Logan et al. 2008). These have been strongly related to the lipid content of the samples (Sweeting et al. 2006, Post et al. 2007,

Abrantes et al. 2011) and for this reason, vary greatly depending on the target animal or the tissue type. For instance, lipid extraction has resulted in changes in δ^{13}C values of 1‰ in fish muscle, up to 2.9‰ in hepatopancreas of spider crabs (*Maja brachydactyla*) and 5.0‰ in fish liver (Sotiropoulos et al. 2004, Sweeting et al. 2006, Bodin et al. 2007). In the present study, lipid extraction from zooplankton and micronekton samples also resulted in a significant increase in δ^{13}C values, although the magnitude of this change varied considerably (0.1 - 1.2‰) among taxa. With the exception of parasitic copepods, these observed shifts were greater than the error associated with analytical variability (± 0.1‰) and average trophic fractionation of δ^{13}C values (0.4‰; Post 2002), which can lead to misinterpretations of food web structure. Typically, lipids are removed from samples when they are presumed to be lipid rich as indicated by C:N ratios > 3.5 (Post et al. 2007). However, the lack of a significant relationship between lipid content and C:N ratios in zooplankton in this study indicates that the use of this threshold might not apply to all species and tissue types. This is further exemplified by C:N ratios well above this level in zooplankton and decapods following lipid extraction, which reflects other carbon compounds still present in the samples. The presence of chitin in exoskeletons or non-lipid energy storage such as glycogen in whole body analyses of invertebrates are likely to mask the relationship between lipid content and C:N ratios (Kiljunen et al. 2006, Logan et al. 2008). Therefore, caution is required when using C:N ratios to infer lipid content from whole body analyses. Extraction of lipids using a polar solvent system, such as the Bligh and Dyer (1959) method as performed in this study, is thus recommended particularly in the case of zooplankton and micronekton to enable comparisons of results within and among studies.

Samples of zooplankton, decapods, fish larvae, mysids and parasitic copepods showed a significant decrease in δ^{13}C values (0.1 – 0.6‰) after acidification, indicating removal of inorganic carbonate. A decrease in C:N ratios was further evidence of elimination of non-dietary carbon. However, this shift in δ^{13}C values was ecologically relevant (> 0.4‰, Post 2002) only in decapods and zooplankton. Similarly, Carabel et al. (2006) and Jaschinsky et al. (2008) found depleted δ^{13}C values in a wide range of marine invertebrates with calcareous body structures and bulk plankton following acidification. In contrast, Pomerleau et al. (2014) reported shifts in δ^{13}C values only for the mollusc *Limacina helicina*, but not for crustacean taxa including copepods, amphipods, euphausiids and chaetognaths. Other studies found no effect of acidification for fish and decapods (Bunn et al. 1995, Bosley and Wainright, 1999; Pinnegar and Polunin, 1999, Mitenbeck et al. 2008). Such contrasting results might reflect high variation in inorganic carbonate within and among taxa (Pomerleau et al. 2014). Different ratios of lipid to inorganic carbonate could also be responsible for the range of outcomes

observed across taxa after the LE+RIC treatment in this study. In order to develop adequate acidification protocols, more studies are required to investigate the relationship between inorganic carbonate and changes in δ^{13}C values in different taxa. Nevertheless, our results indicate the need for acidification of tissue of organisms containing inorganic carbonate (e.g. exoskeleton or calcareous structures), notably for samples of zooplankton and decapods.

The effects of LE and RIC treatments on zooplankton and micronekton were more unpredictable for δ^{15}N than for δ^{13}C values and for this reason, caution is required when analyzing samples for nitrogen isotopes. The δ^{15}N values of all taxa were significantly affected by lipid extraction, although to varying amounts (-0.2 – 0.8‰). Increases in values were found only for euphausiids, fish larvae and mysids. These conflicting results support the diverse range of effects of lipid extraction on δ^{15}N values observed by previous studies, which include changes of 0.15‰ for zooplankton (Syväranta and Rautio, 2010) and 2.7‰ for whole juvenile fish (Sotirpoulos et al. 2004). The effects of acidification treatment on δ^{15}N values also varied among taxa, with only zooplankton and cephalopods showing significant shifts of 0.6‰ and 0.2‰, respectively. Outcomes of acidification treatments also differed among studies, with some reporting no effect (Bosley and Wainright, 1999, Carabel et al. 2006, Jaschinski et al. 2008), while others have shown increases (Pinnegar and Polunin, 1999) and even decreases (Jacob et al. 2005) in δ^{15}N values for a variety of fishes and marine invertebrates.

The greatest changes in values of δ^{15}N (-0.3 – 1.1‰) were observed after RIC+DIW treatment. The increase in C:N ratios in all taxa confirmed that rinsing with deionized water further leaches nitrogen compounds (Bunn et al. 1995, Bosley and Walnright, 1999, Jaschinski et al. 2008). Although the changes in δ^{15}N values associated with LE, RIC, LE+RIC and RIC+DIW were below the estimated enrichment of 3.4‰ between trophic levels (Post 2002), thus might not alter overall interpretation of food webs, it is likely to reduce the accuracy of trophic calculations. For this reason, future studies should refrain from lipid extraction, acidifying for nitrogen and washing with deionized water and consequently, two sub-samples of each sample will be required for SIA; one treated (LE, RIC or LE+RIC) for the analysis of carbon and another untreated for the analysis of nitrogen.

The chemistry behind shifts in δ^{15}N values after LE and RIC treatments are still unclear. Goering et al. (1990) suggested that changes in δ^{15}N values after acidification might be caused by the leaching of organic nitrogen compounds (e.g. amino acids, nucleic acids) with different δ^{15}N values. It is known, for example, that non-essential amino acids tend to accumulate more ^{15}N than essential amino acids (McClelland and Montoya, 2002, Schmidt et al. 2004). The potential removal of lipoproteins by chloroform:methanol could have the same impact on δ^{15}N

values (Sotiropoulos et al. 2004, Sweeting et al. 2006, Bodin et al. 2007). However, other studies proposed that the observed changes after lipid extraction could be a result of the removal of isotopically-light nitrogenous waste (ammonia and ammonium) remaining in tissues (Murry et al. 2006, Ingram et al. 2007, Mitenbeck et al. 2008), as was the case for the whale shark sub-dermal tissue in this study. If ^{15}N-depleted compounds were removed from samples, consistent increases in δ^{15}N values would be predicted. This did not occur in this study, indicating that compounds other than ammonia or ammonium were also extracted. Further studies are needed to determine the mechanisms by which treatments affect values of nitrogen compounds prior to analysis. If, for example, ammonia is present in lipid-extracted water rinses, this needs to be removed from samples as it can bias trophic level interpretations.

3.4.3. Mathematical normalization

Mathematical normalizations have been proposed as a reliable tool to remove known sources of variability such as those resulting from the influence of lipids, urea and inorganic carbonate in SIA (Post et al. 2007, Logan et al. 2008). However, generic correction models might not be suitable for all species and tissues (Abrantes et al. 2011), as our results suggested. Only mathematical normalizations by Li et al. (2016) developed from lipid extracted δ^{13}C values and Churchill et al. (2015) developed from muscle tissue of *Centrophorus cf. granulosos* predicted isotopic values of sub-dermal tissue of whale sharks. Differences in the chemical structure and concentrations of lipid and urea among species and tissues hinders the ability to exchange correction models among studies. In addition, the confounding effects of urea and lipids in elasmobranch tissues (Carlisle et al. 2016) reinforces the need for more knowledge of isotope dynamics before mathematical corrections can be applied with confidence.

Similarly, models for lipid correction did not predict accurate δ^{13}C values for zooplankton and micronekton, with the exception of those by McConnaughey and McRoy (1979) and Logan et al. (2008) for fish larvae and decapods, respectively. Overall, these models were based on the observed relationships between C:N ratios and lipid content and between lipid content and shifts in δ^{13}C values with lipid removal (e.g. Post et al. 2007, Abrantes et al. 2011). However, C:N ratios might not be a reliable proxy of lipid content and changes in δ^{13}C values in all taxa and tissues, as was shown to be the case for zooplankton in this study. The relationship between the RIC treatment and shifts in δ^{13}C values was also unclear, although our results suggested that corrections for inorganic carbonate in zooplankton and decapod samples could be obtained using the model of Pomerleau et al. (2014). This was not necessarily the case for

other taxa. Future studies should investigate the effects of lipids and inorganic carbonate on C:N ratios and $\delta^{13}C$ values before generalized correction models can be applied. Thus, the most robust approach to reduce bias is to perform extractions (e.g. lipids, urea and inorganic carbonate) from samples whenever possible, or alternatively to develop specific correction models using a subset of the samples, provided that clear relationships are present (Fig. 3.2).

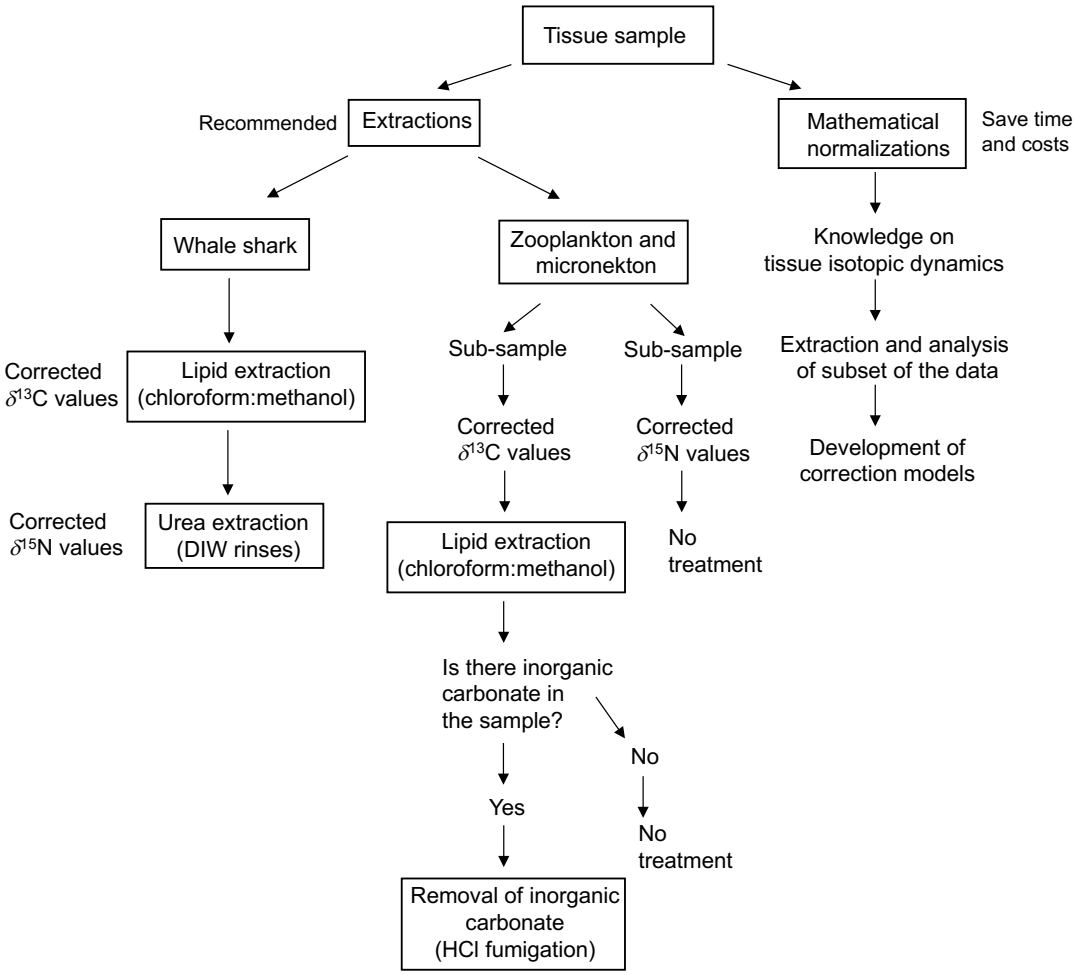


Figure 3.2. Schematic diagram of the protocol to prepare whale shark sub-dermal tissue and zooplankton and micronekton taxa prior to stable isotope analysis of carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values). DIW = deionized water.

3.5. Conclusions

Results in this study indicate that lipid and urea content do need to be taken into account in the analysis of δ^{13}C and δ^{15}N values in tissues of elasmobranchs. The LE+DIW treatment was the most efficient method to remove both lipid and urea and is therefore recommended as the most appropriate technique for this task. For zooplankton and micronekton samples, two separate aliquots should be used: one for the analysis of δ^{13}C treated with LE (to remove lipids) or LE+RIC, acidification if samples contain inorganic carbonate (e.g. exoskeletons or calcareous structures), with another portion left untreated for the analysis of δ^{15}N values. The reason(s) for the variable effects of LE and RIC treatments on δ^{15}N values observed in this study are still unclear. Future research should examine the mechanisms of chloroform:methanol (and other solvents) extraction and acidification in the removal of nitrogen compounds. Published mathematical normalizations should be used with caution and may not be appropriate for all species or tissue types. Species and tissue-specific models for corrections should be developed during the process of analysis, but will require knowledge of the isotopic dynamics of sampled tissues.

Chapter 4

The trophic ecology of whale sharks on a fringing coral reef from stable isotope analysis

Marcus L, Virtue P, Nichols PD, Ferreira L, Pethybridge H, Meekan MG. The trophic ecology of whale sharks on a fringing coral reef. *In preparation*

Abstract

Using stable isotope analysis (δ^{13}C and δ^{15}N values) this study investigated the trophic ecology of whale sharks (*Rhincodon typus*) biopsied at Ningaloo Reef, Western Australia, in 2013 and 2014. We examined trophic relationships of whale sharks with the local food web and assessed variations in their feeding habits in relation to their size and sex. These sharks (mainly juvenile males, 3 – 8.5 m total length TL) had a trophic position estimate of 2.6, similar to planktivorous and omnivorous reef fishes. The ^{13}C signature of *R. typus* ranged from -15.7‰ to -13.3‰, consistent with these animals being part of food chains based on inshore benthic sources of carbon. These results imply that demersal zooplankton is potentially an important component of the diet of whale sharks in reef systems. In contrast, the δ^{13}C values of zooplankton and nektonic taxa collected at Ningaloo Reef ranged from -18.9‰ to -16.5‰, suggesting that these taxa were part of food chains based on pelagic sources of carbon. Values of δ^{15}N (from 6.9‰ to 10.8‰) increased with body size of whale sharks, a pattern more pronounced in females. Such ontogenetic changes possibly result from a transition from offshore to more coastal habitats as whale sharks grow (3 – 8 m). This study provides further evidence of size- and sex-specific patterns of feeding in whale sharks, which is key knowledge for a better implementation of conservation strategies.

4.1. Introduction

Of the ~500 extant species of shark, the whale shark (*Rhincdon typus)* is the largest and one of only three to evolve filter-feeding as a means to gather food. Similar to baleen whales (Werth 2000, Potvin et al. 2012), whale sharks can attain giant body sizes because this mode of feeding allows them to target and efficiently gather abundant planktonic prey that are many orders of magnitude smaller than their own body size (Meekan et al. 2015). This food is, however, patchy both in space and time and as a result, whale sharks are highly mobile (Wilson et al. 2006, Hueter et al. 2013, Hearn et al. 2016), occupying both coastal and open-ocean habitats. Similar to many other sharks (Wetherbee and Cortés, 2004, Wearmouth and Sims, 2008), populations are segregated by size and sex, so that juvenile males are often encountered in seasonal aggregations at near-shore locations in tropical and warm-temperate locations worldwide, whereas females, pups (< 3 m) and adults (> 8 - 9 m) are thought to occupy deeper, oceanic waters (Meekan et al. 2006, Rowat et al. 2008, Rohner et al. 2015, Ramírez-Macías et al. 2017). These patterns suggest that the foraging of these sharks and thus their role in oceanic and coastal ecosystems is likely to vary both in space and time.

In Australian waters, seasonal aggregations of whale sharks (mostly juvenile males < 8 m total length) occur between March and June every year at Ningaloo Reef, Western Australia (Meekan et al. 2006), where the continental shelf reaches its narrowest point (~10km). During the austral autumn, the presence of these large zooplanktivores along the reef slope has been associated with an increase in planktonic productivity close to the reef that is driven by an acceleration of the southward-flowing Leeuwin Current offshore (Wyatt et al. 2012a). At this locality, whale sharks have been observed feeding on euphuasiid swarms (*Pseudeuphausia latifrons)*, crab megalopae, chaetognaths, copepods, stomatopod larvae and schools of small fish at the surface (Wilson et al. 2001a, Jarman and Wilson, 2004, Taylor 2007). However, recent biochemical studies of these sharks at both Ningaloo and off the coast of Mozambique suggested that prey from coastal benthic and meso-pelagic habitats such as demersal zooplankton or other prey in the deep-scattering layer are also key components of the diet (Couturier et al. 2013b, Rohner et al. 2013, Marcus et al. 2016). These conflicting observations mean that at present, it is difficult to determine the role of whale sharks in coastal ecosystems and the degree to which they act as links between oceanic and reef environments.

The analysis of stable isotopes has been established as an effective means to investigate the trophic ecology of sharks (e.g. Papastamatiou et al. 2010, Abrantes and Barnett, 2011, Albo-Puigserver et al. 2015). The isotopic composition of an animal's tissue reflects assimilated diet over time, with the conservative increase in δ^{13}C values from prey to consumer indicating the

food web source of the diet, and the fractionation in δ^{15}N values providing an estimate of trophic level (Peterson and Fry, 1987, Post 2002). Local biogeochemical processes in the marine environment lead to temporal and spatial variations in the isotopic composition of the base of the food web across ocean basins (François et al. 1993, Goericke and Fry, 1994, Montoya 2007). This isotopic variation in food webs would be recorded in tissue of consumers depending on their movement patterns and foraging behavior (Popp et al. 2007, Graham et al. 2010). As a result, inshore/offshore and pelagic/benthic differences in the structure of food chains can be relatively straightforward to detect (Graham et al. 2010, Hobson et al. 2010).

Studies using stable isotope techniques are now common place and in many localities they have generated data bases of isotopic composition of animals sampled throughout the food web, from primary consumers to apex predators, as for example at Ningaloo Reef (Speed et al. 2012, Wyatt et al. 2012b, 2013, Ferreira et al. in press). Although differences in methodological approaches among studies must be acknowledged (Marcus et al. 2017), this archive is very useful because it potentially allows targeted studies on particular species or guilds of consumers to be placed in the context of the wider food chain. Here, we investigate the feeding ecology and trophic niche of whale sharks aggregating at Ningaloo Reef using stable isotope analysis. Published isotopic studies were used to define and compare the role of whale sharks with those of other major guilds in the fish assemblage. These results were also compared with analyses of the zooplankton and nektonic communities sampled at Ningaloo that are thought to form some of the components of the diet of whale sharks. In order to examine intraspecific differences in habits of feeding and movement patterns of sharks, whale sharks were sampled in the same months in two different years (2013 and 2104) across individuals of different sizes and both sexes.

4.2. Materials and Methods

4.2.1. Collection of samples

Biopsies were sampled from 50 different individual whale sharks at Ningaloo Reef (Western Australia; 22° 33′ 45″ S, 113° 48′ 37″ E) during May 2013 and May 2014, coinciding with the annual aggregation of whale sharks at this location. Two centimeters of skin and sub-dermal tissue was collected from the left side of the animals using a hand spear fitted with a biopsy probe tip and applied by a snorkeler. A third of the biopsy, without the skin, was immediately frozen in liquid nitrogen for SIA. Whale sharks were sexed according to the absence or presence of claspers in their pelvic fins. Biopsies were collected from 19 males and 5 females

in 2013 and from 20 males and 6 females in 2014. Total length (TL) of whale sharks was estimated to range from 3 to 8.5 m for males and from 3 to 7 m for females (Appendix 4.1).

Potential prey of whale sharks at Ningaloo Reef included zooplankton (100 – 1000 µm) and a wide range of other nektonic taxa (> 1000 µm) such as decapod and stomatopod larvae, euphausiids, mysids, amphipods, isopods, decapods (adult), polychaetes, fish larvae and pelagic fish. Zooplankton samples, stomatopod and decapod larvae were collected both in 2013 and 2014, whereas other nekton were sampled only in 2014 (for species details see Marcus et al. 2016). Samples were collected at different stations within 10 km offshore of the reef front of the fringing reef at Ningaloo (see Marcus et al. 2016 for map and details of collection). Zooplankton samples were collected by horizontal or oblique tows using 200 and 300 µm mesh nets for 10 min at a speed of ~2 knots from a boat. Other nekton taxa were sampled using two light traps (see Meekan et al. 2001 for design) deployed at the surface during the night. All samples were transported in insulated containers with ambient sea water to shore. Once in the laboratory, a Folsom's Sample Divider was used to divide zooplankton samples. A quarter of the sample was kept frozen for SIA and another quarter was fixed with 70% ethanol for further taxonomic identification. Samples from light trap collections were sorted to the highest taxonomic resolution and a few representatives of each taxa were frozen.

4.2.2. Lipid and urea effects on whale shark isotopic signatures

Prior to analysis, whale shark samples were lipid- and urea-extracted to standardize δ^{13}C and δ^{15}N values. Since lipids are depleted in ^{13}C relative to protein and carbohydrates (DeNiro and Epstein, 1977), their presence potentially affects δ^{13}C values of sub-dermal tissue of whale sharks. Similarly, sharks retain ^{15}N-depleted nitrogenous compounds (e.g. urea and trimethylamine oxide), which may result in artificially lower δ^{15}N values (Marcus et al. 2017). A combination of a standard chloroform:methanol:water extraction followed by deionized water rinsing was used to remove both lipids and urea as following protocols of Marcus et al. (2017).

4.2.3. Stable isotope analysis

All samples (sub-dermal tissue of whale sharks, zooplankton and nekton taxa) were freeze-dried and ground to a fine powder. Approximately 0.4 to 0.7 mg of samples were weighed in tin capsules and stable isotopes analyzed using a flash combustion isotope ratio mass spectrometry (varioPYRO cube coupled to Isoprime100 mass spectrometer) at the Central Science Laboratory, University of Tasmania (Australia). The results are expressed in delta (δ)

values as the deviations from conventional standards in parts per thousand (‰) from the following equation:

(1) $$\delta X = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

where X = ^{13}C or ^{15}N and R = the ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Reference standards for quantifying ^{13}C and ^{15}N materials were Pee Dee Belemnite (PDB) and atmospheric nitrogen, respectively. Stability of the instrumentation, analytical precision, drift correction and linearity performance were calculated from the repetitive analysis of these standards. The method precision was ± 0.1‰ for both isotopes.

4.2.4. Lipid effects on zooplankton and nektonic taxa isotopic signatures

Lipids can also affect $\delta^{13}C$ values of zooplankton and nekton taxa in this study (Marcus et al. 2017). The following lipid normalization equations were applied whenever appropriate to adjust $\delta^{13}C$ values (Marcus at al. 2017):

Zooplankton, euphausiids, mysids, amphipods, isopods and polychaetes:

(2) $$\delta^{13}C_{LE} = -12.44 + 0.33 \times \delta^{13}C_{bulk}$$

where $\delta^{13}C_{LE}$ was the value of $\delta^{13}C$ after lipid normalization and $\delta^{13}C_{bulk}$ was the direct measurement of $\delta^{13}C$ of the target animal. This equation was developed from whole body zooplankton samples collected at the same time and place as the present study (see Marcus et al. 2017).

Fish larvae:

(3) $$L = 93 / [1 + (0.246 \times C{:}N - 0.775)^{-1}]$$

(4) $$\delta^{13}C_{LE} = \delta^{13}C_{bulk} + D\,[\,I + 3.9 / (1 + 287 / L)]$$

where L was lipid content, D was 7.018‰, the difference in isotopic composition between protein and lipid, and I was a constant value of 0.048. Equation by McConnaughey and McRoy (1979) and revised by Kiljunen et al. (2006).

Decapods:

(5) $\delta^{13}C_{LE} = \delta^{13}C_{bulk} + \beta_0 + \beta_1 \ln (C:N)$

where β_0 and β_1 were estimated parameters for different animal taxa (Logan et al. 2008).

4.2.5. Trophic position and Ningaloo food web connections to whale sharks

The trophic position of whale sharks was estimated using the equation of Post (2002):

(6) $TP = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{base}) / \Delta^{15}N$

where λ was the trophic position of the selected baseline organism, $\delta^{15}N_{consumer}$ was the $\delta^{15}N$ value of whale sharks (mean of whale sharks for 2013 and 2014, $\delta^{15}N$ = 9.2‰), $\delta^{15}N_{base}$ was the $\delta^{15}N$ value of the baseline organism in the food web and $\Delta^{15}N$ was the trophic discrimination factor between trophic levels. Zooplankton samples of 100 µm, filter size, (phytoplankton feeders, TP = 2) were used as baseline organisms with $\delta^{15}N$ values ($\delta^{15}N_{base}$) of 5.9‰ (mean of seven samples). To enable accurate estimations of the trophic position of a species within the food web, species-specific tissue discrimination factors ($\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{prey}$) were required. Since $\Delta^{15}N$ has not been determined for sub-dermal tissue of any planktivorous elasmobranch, a $\Delta^{15}N$ of 5.1‰ was used according to a scaled $\Delta^{15}N$ framework developed from muscle tissue of zooplanktivores, including whale sharks from South Africa, by Hussey et al. 2014. In addition, the widely used $\Delta^{15}N$ of 3.4‰ by Post 2002, was also applied for further comparison.

The isotopic composition of whale sharks was plotted against other taxa at Ningaloo Reef extracted from published literature including primary producers, other consumers and predators (Speed et al. 2012, Wyatt et al. 2012b, 2013, Ferreira et al. in press). Differences in isotopic values of zooplankton samples, stomatopod and decapod larvae between years of collection, were compared using a T-student test or a Wilcoxon-Mann-Whitney Test when assumptions of normality and homogeneity of the variance were not met. The assumption of normality was verified using a Shapiro-Wilk Test and the homogeneity of the variances was analyzed with a Bartlett Test. We also visually compared the isotopic signatures of whale sharks and their potential prey at Ningaloo Reef by plotting the average values of $\delta^{13}C$ and $\delta^{15}N$ of each group.

4.2.6. Isotopic variance of whale sharks

Generalized linear models (GLM) with a Gaussian distribution were used to assess how much variation in δ^{13}C and δ^{15}N values of whale shark sub-dermal tissue could be explained by year, sex and total length (TL). Full subsets of all combinations of the set of predictors were fitted with the package *MUMIn* (Barton 2013). Model selection was performed by ranking the models by the Akaike's Information Criterion with a second-order correction for small sample size (AICc) (Burnham and Anderson, 2002). AICc values were also presented as AICc differences (ΔAICc) and the AICc weights (wAICc). Goodness of fit was assessed by the percentage deviance explained (%DE). The R-package visreg (Breheny and Burchett, 2016) was used to plot the top-ranked models.

4.3. Results

4.3.1. Trophic position and Ningaloo food web connections to whale sharks

The raw isotopic values of sub-dermal tissue of whale sharks in 2013 and 2014 are summarized in Table 4.1. The average of trophic position of whale sharks was 2.6 ± 0.0‰ when using a DTDF of 5.1‰ (Hussey et al. 2014) and 2.9 ± 0.0‰ when using a DTDF of 3.4‰ (Post 2002). Ratios of C:N ranged from 2.6 to 3.1. Among individuals, δ^{13}C and δ^{15}N values ranged from -15.7‰ to -13.3‰ and from 6.9‰ to 10.8‰, respectively (Appendix 4.1). Large differences (1.2‰) in δ^{13}C values were observed among males of ≥ 8 m TL between years (Table 4.1). Females of ≤ 4 m TL showed the largest difference in δ^{15}N values (1.1‰) between years (Table 4.1).

The isotopic composition of whale sharks was plotted with those of other fishes, sharks and primary producers at Ningaloo Reef (Fig. 4.1). Values of δ^{13}C in whale sharks (mean of -15.1 ± 0.3 in 2013 and -14.9 ± 0.0 in 2014) were similar to those of coral mucus and macroalgae, whereas δ^{15}N values (mean of 9.4 ± 0.2 in 2013 and 9.0 ± 0.2 in 2014) were similar to herbivorous and planktivorous reef fishes. Whale sharks occupied a lower trophic position than other species in the reef shark assemblage.

Table 4.1. The mean isotopic values (‰ ± standard error) of whale shark sub-dermal tissue and potential prey collected at Ningaloo Reef in May 2013 and 2014.

Year	Group	n	δ^{13}C (‰)	δ^{13}C (‰) normalized	δ^{15}N (‰)	C:N
2013	Whale shark[a]	24	-15.1 ± 0.3	- ± -	9.4 ± 0.2	2.8 ± 0.0
	Male[b] (3 – 8.5 m)	19	-15.0 ± 0.2	- ± -	9.4 ± 0.2	2.8 ± 0.0
	Female[b] (4 – 6.5 m)	5	-15.3 ± 0.1	- ± -	9.1 ± 0.5	2.9 ± 0.0
	Zooplankton[c]	10	-19.7 ± 0.3	-18.9 ± 0.1	5.9 ± 0.4	4.2 ± 0.1
	Decapod larvae[d]	5	-18.9 ± 0.2	-17.5 ± 0.2	4.7 ± 0.1	6.2 ± 0.3
	Stomatopod larvae[d]	4	-18.8 ± 0.2	-17.3 ± 0.4	7.4 ± 0.4	6.8 ± 0.9
	Spratelloides spp[e]	3	-17.5 ± 0.6	-16.5 ± 0.4	7.4 ± 0.2	3.5 ± 0.2
2014	Whale shark[a]	26	-14.9 ± 0.0	- ± -	9.0 ± 0.2	2.9 ± 0.0
	Male[b] (3 – 8 m)	20	-14.9 ± 0.1	- ± -	9.0 ± 0.2	2.9 ± 0.0
	Female[b] (3 – 7 m)	6	-14.9 ± 0.3	- ± -	9.1 ± 0.5	2.9 ± 0.0
	Zooplankton[c]	12	-18.7 ± 0.3	-18.6 ± 0.1	6.6 ± 0.4	4.3 ± 0.2
	Decapod larvae[d]	6	-18.2 ± 0.3	-17.1 ± 0.2	6.1 ± 0.3	5.4 ± 0.5
	Stomatopod larvae[d]	4	-19.3 ± 0.1	-17.8 ± 0.4	9.0 ± 0.1	6.9 ± 1.2
	Euphausiids[c]	4	-19.3 ± 0.1	-18.8 ± 0.0	7.9 ± 0.3	6.7 ± 0.7
	Mysids[c]	5	-17.8 ± 0.1	-18.3 ± 0.0	8.7 ± 0.1	6.9 ± 1.1
	Amphipods[c]	6	-18.1 ± 0.3	-18.4 ± 0.0	9.1 ± 0.3	6.6 ± 0.7
	Isopods[c]	4	-18.4 ± 0.6	-18.5 ± 0.7	9.0 ± 0.3	6.0 ± 0.2
	Decapods[d]	5	-18.6 ± 0.4	-17.9 ± 0.1	8.6 ± 0.3	4.8 ± 1.1
	Polychaetes[c]	4	-19.2 ± 0.3	-18.8 ± 0.1	6.5 ± 0.1	9.1 ± 1.9
	Fish larvae[e]	23	-19.0 ± 0.2	-17.3 ± 0.1	8.5 ± 0.2	4.0 ± 0.1
	Pelagic fish[e]	3	-17.8 ± 0.4	-17.0 ± 0.0	10.9 ± 0.2	3.4 ± 0.2

[a] Isotopic mean values of whale sharks according to year only

[b] Isotopic mean values of whale sharks according to sex only

[c] Normalized for lipids according to Equation 10

[d] Normalized for lipids according to Logan et al. (2008)

[e] Normalized for lipids according to Kiljunen et al. (2006)

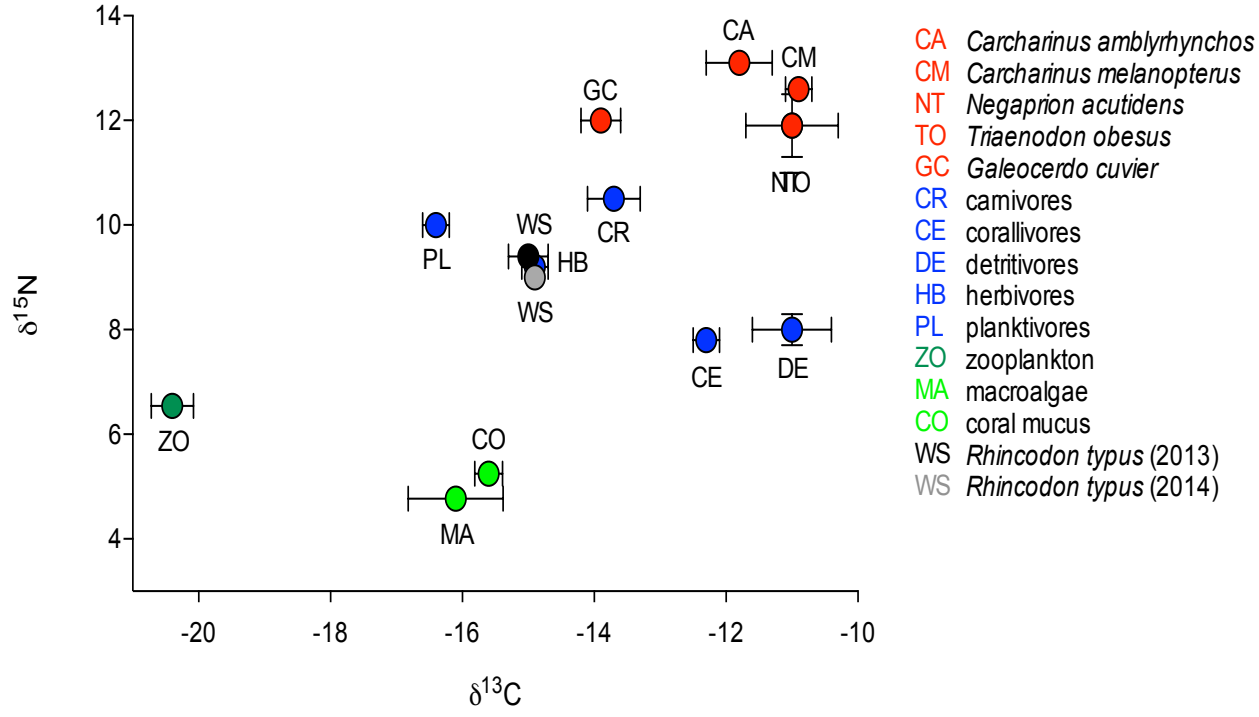


Figure 4. 1. Biplot of mean isotopic values (‰ ± standard error) of whale sharks (this study), other consumers and primary producers at Ningaloo Reef (data from Speed et al. 2012, Wyatt et al. 2012b, 2013 and Ferreira et al. in press.). Red = sharks, blue = fish community, dark green = zooplankton, light green = producers, black/grey = whale sharks.

Groups of potential prey collected at Ningaloo were separated by year due to significant differences in isotopic values. This included annual differences in δ^{13}C values of zooplankton ($W = 22$, $p = 0.013$) and δ^{15}N values in decapod larvae ($t = -4.764$, $df = 5.303$, $p < 0.01$) and stomatopod larvae ($t = -4.079$, $df = 6$, $p < 0.01$). The ^{13}C signatures of whale sharks were more enriched than zooplankton and other nekton taxa (Table 4.1, Fig. 4.2). Overall, zooplankton samples for both years, polychaetes, euphausiids, isopods, amphipods and mysids all had depleted ^{13}C signatures < -18‰ (Table 4.1, Fig. 4.2). Intermediate δ^{13}C values of -17.8‰ ± 0.4 and -17.9‰ ± 0.1 were recorded for stomatopod larvae collected in 2014 and decapods, respectively. More enriched ^{13}C signatures were characteristic of decapod larvae for both years, stomatopod larvae for 2013, fish larvae and pelagic fishes. Values of δ^{13}C for these groups ranged from -17.5‰ to -17.0‰. *Spratelloides* spp. showed signatures that were more enriched in ^{13}C than any other prey taxa -16.5‰ ± 0.4 (Table 4.1, Fig. 4.2).

The ^{15}N signatures of whale sharks were similar to some nekton taxa including isopods, amphipods, stomatopod larvae in 2014, mysids, decapods and fish larvae. Values of $\delta^{15}N$ for these prey groups ranged from 8.5‰ to 9.1‰ (Table 4.1, Fig. 4.2). More depleted ^{15}N signatures were observed for euphausiids, stomatopod larvae in 2013, *Spratelloides* spp., zooplankton for both years, polychaetes and decapod larvae in 2014 with $\delta^{15}N$ values ranging from 7.9‰ to 5.9‰. Decapod larvae collected in 2013 had the most depleted ^{15}N signature (4.7 ± 0.1‰), whereas pelagic fish had the most enriched (10.9 ± 0.2‰, Table 4.1, Fig. 4.2). The C:N ratios ranged from 3.4 in pelagic fish to 9.1 in polychaetes (Table 4.1).

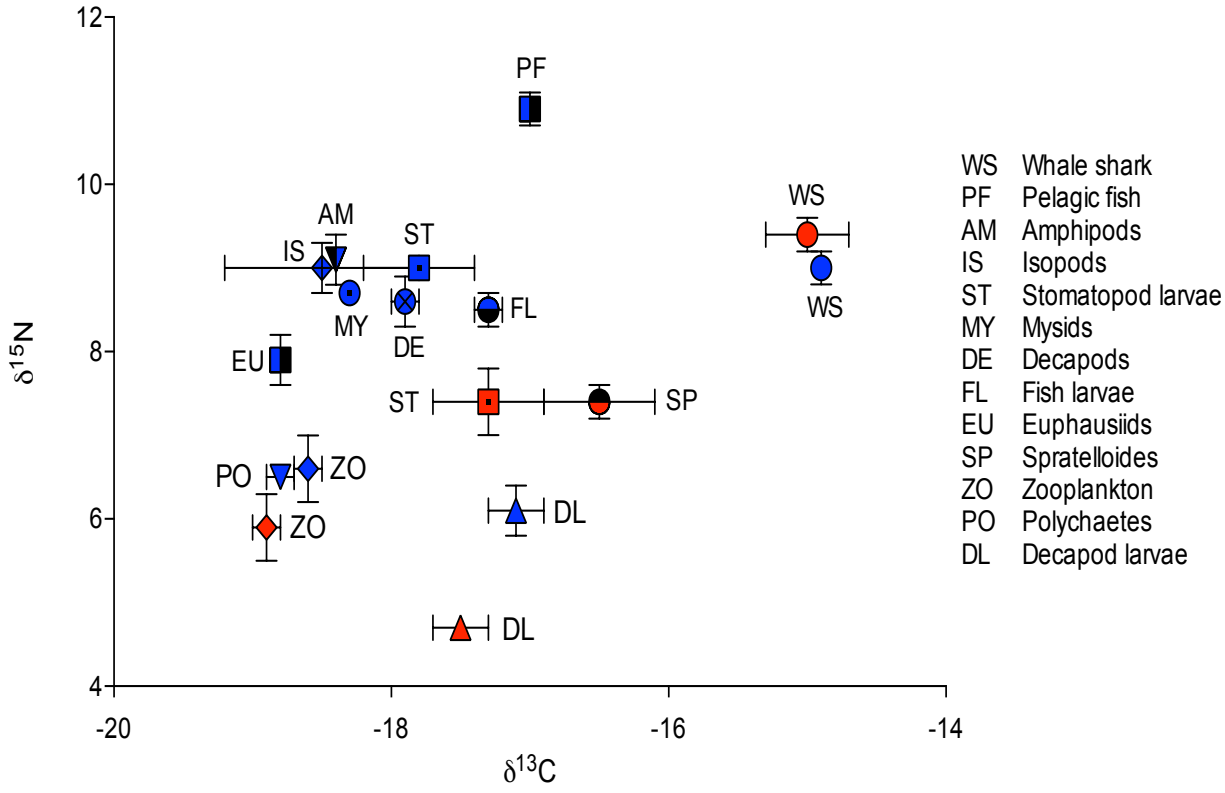


Figure 4. 2. Biplot of mean isotopic values (‰ ± standard error) of whale sharks and potential prey (normalized for lipids) collected at Ningaloo Reef. Red = 2013, blue = 2014.

4.3.2. Isotopic variance of whale sharks

The model selected to explain variance in δ^{13}C values in sub-dermal tissue of whale sharks included total length (TL). Although this variable only explained 6% of the deviance in the response (Table 4.2), the model indicated an increase in δ^{13}C values with size (Fig. 4.3a). For values of δ^{15}N, the selected model included total length (TL) and sex, and the interaction between these two terms, and explained 33% of the deviance (Table 4.2). Model predictions showed an increase in δ^{15}N values with size, which was more pronounced in females than in males (Fig. 4.3b).

Table 4.2. Ranked Generalised Linear Models for δ^{13}C and δ^{15}N values for whale shark sub-dermal tissue. Values in bold indicate the top ranked model according to sample corrected Akaike's Information Criterion (AICc), AIC differences (ΔAICc), AICc weights and percentage of deviance (%DE).

Response	Model	df	AICc	ΔAICc	wAICc	%DE
δ^{13}C	**length**	**3**	**89.9**	**0.0**	**0.3**	**6.0**
	length + year	4	91.1	1.2	0.2	8.3
	length + sex	4	92.1	2.2	0.1	6.4
	year	3	92.2	2.3	0.1	1.6
	length + year + length x year	5	92.6	2.7	0.1	10.1
	sex	3	92.6	2.7	0.1	0.8
δ^{15}N	**length + sex + length x sex**	**5**	**128.4**	**0.0**	**0.4**	**32.7**
	length + sex + year + length x sex	6	129.8	1.4	0.2	34.3
	length	3	130.4	2.0	0.2	22.9
	length + year	4	131.7	3.3	0.1	24.5
	length + sex	4	132.7	4.3	0.0	22.9
	length + year + length x year	5	133.3	4.9	0.0	25.9

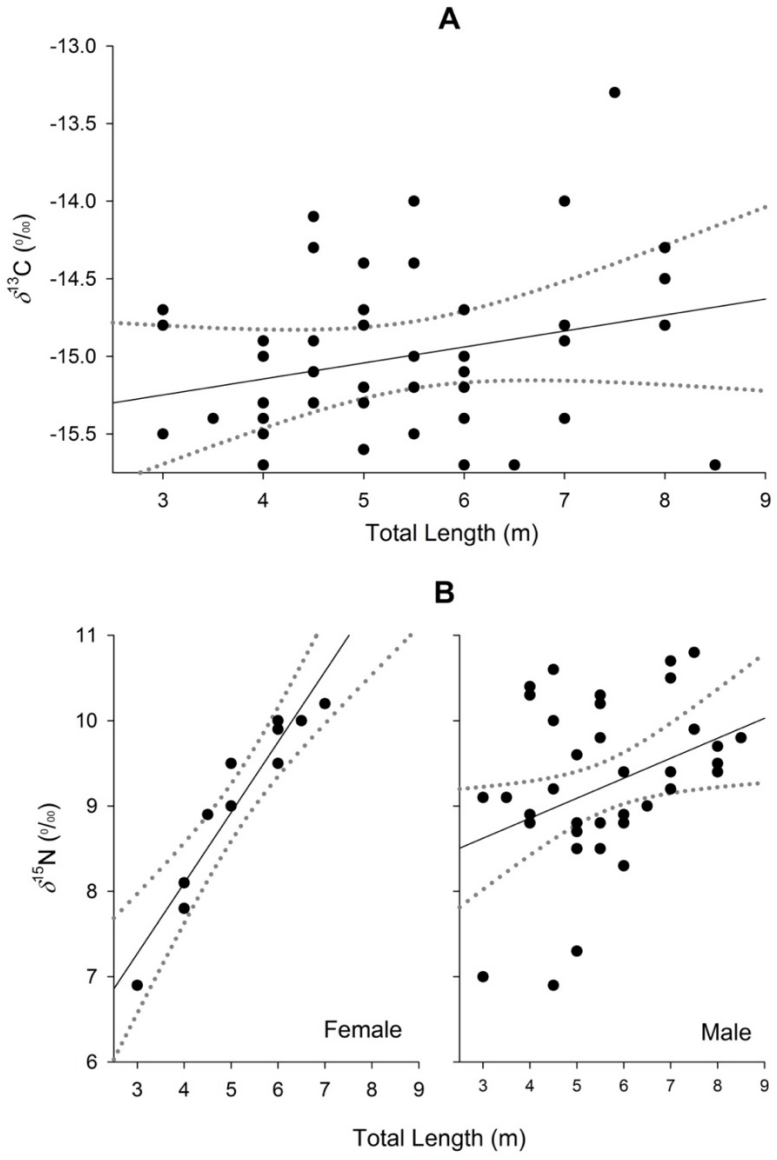


Figure 4. 3. Partial dependence plots of the relationship between $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values and the explanatory variables for the top-ranked models from analysis of whale shark sub-dermal tissue. Dashed lines represent 99% confidence interval.

4.4. Discussion

The isotopic signatures of whale sharks sampled at Ningaloo Reef were not consistent with those of an epipelagic species feeding predominantly on surface zooplankton and nekton, as has been suggested by direct observations both at Ningaloo and at other aggregations (e.g. Clark and Nelson, 1997, Heyman et al. 2001, Taylor 2007), or through the analysis of stable isotopes in other locations (Borrell et al. 2011 a,b). Indeed, whale sharks appeared to occupy a similar trophic position to planktivorous reef fishes at Ningaloo that were feeding within inshore benthic food webs. We also observed intraspecific differences in diet according to size and sex, although the aggregation of sharks at Ningaloo Reef was dominated by juvenile (length range 3 – 8.5 m TL) males (male:female sex ratio, 4 : 1), a pattern consistent both with earlier studies and with observations at other aggregation sites world-wide (Meekan et al. 2006, Rowat et al. 2011, Rowat and Brooks, 2012, Araujo et al. 2017).

4.4.1. Feeding of whale sharks at Ningaloo Reef

Our mean estimate of trophic position of whale sharks (2.6 and 2.9 using a DTDF of 5.1‰ and 3.4‰, respectively) were lower than those based on analysis of stomach contents of individuals from South African and Indian waters (TP = 3.6, Cortés 1999) and isotope analyses of muscle of whale sharks collected by fisheries in the Arabian Sea, off the coast of India (TP = 3.3, Borrell et al. 2011b). These differences might arise from the use of a scaled $\Delta^{15}N$ framework by our study (Hussey et al. 2014), but are also likely a result of whale sharks at Ningaloo feeding at a more basal level of $\delta^{15}N$ values than individuals in the Arabian Sea. It is also important to note that the Indian fishery occurred many kilometers from the coast along a relatively wide continental shelf in a highly polluted area (Borrell et al. 2011a), whereas the sharks at Ningaloo were sampled close to a fringing coral reef on a narrow shelf. Such differences in habitat are likely to be accompanied by different prey fields for whale sharks in each location.

In both 2013 and 2014, whale sharks at Ningaloo Reef occupied a trophic position very similar to the herbivorous and planktivorous reef fishes sampled by Wyatt et al. (2012b). To a large extent, this result can be explained as a consequence of the very broad trophic categories applied by Wyatt et al. (2012b), who included samples of damselfishes that, although nominally herbivorous, fed on a predominantly planktivorous diet in the reef front habitat where the majority of individuals were collected for their study. Additionally, Wyatt et al (2012b) also included acanthurids that were largely detritivores in the herbivore group. As expected, the $\delta^{15}N$ values of whale sharks were higher than zooplankton, euphausiids, decapod larvae,

small fish (*Spratelloides* sp.) and other invertebrates, many of which are known prey items (Jarman and Wilson, 2004, Rowat and Brooks, 2012, Rohner et al. 2013). Caution is required when interpreting this data however, as some nektonic taxa, notably decapod and stomatopod larvae, displayed large differences in values of δ^{15}N between years, which might have reflected changes in upwelling regimes along the coast (Wyatt et al. 2012b).

Values of δ^{13}C in the marine environment typically increase from pelagic waters offshore to productive nearshore benthic ecosystems (France 1995, Hobson et al. 1997, Abrantes and Barnett, 2011). High δ^{13}C values of whale sharks thus indicated a strong dependency on inshore benthic food webs. These results were not consistent with those of Borrell et al. (2011 a,b), who found that the isotopic composition of whale sharks (size range 3 – 18.8 m TL) caught in the Arabian Sea off the coast of India indicated a diet of pelagic zooplankton that was very similar to that of clupeid fishes (notably *Ilisha melastoma*). Such differences in the diet and role of sharks across a species distribution appear to be commonplace and have been observed previously in tiger (*Galeocerdo cuvier*), sevengill (*Notorinchus cepedianus*) and blacktip reef (*Carcharinus melanopterus*) sharks, a pattern thought to reflect differences in prey availability, the local environment and the sex or sizes of the animals sampled by independent studies (Papastamatiou et al. 2010, Abrantes and Barnett, 2011, Ferreira et al. in press). Unlike the present study, which sampled mainly juvenile males, the work of Borrell et al. (2011 a,b) in the Arabian Sea largely focused on females that tended to be much larger than the animals found at Ningaloo. Adult animals are likely to largely reside in oceanic waters (Meekan et al. 2015), and thus feed in environments that are relatively depleted in carbon. This seems a likely explanation of the differences in results between Borrell et al's (2011 a,b) work and the present study.

Whale sharks reflected a diet based on prey from inshore benthic food webs associated with macroalgal or reef-derived producers at Ningaloo (Wyatt et al. 2012b, 2013). Accidental (or incidental) ingestion of drifting macroalgae is possible, since small pieces of the brown seaweed *Sargassum* sp., have been found in large quantities in the stomach contents of whale sharks in the Indian Ocean (Rohner et al. 2013). This seaweed forms dense slicks, windrows and mats in surface waters at Ningaloo Reef during the time that whale sharks were sampled. Whale sharks at Ningaloo have been observed surface ram filter-feeding through these algal mats (pers. obs.). However, the biochemical/isotopic link between whale sharks and reef systems is likely to occur through the ingestion of demersal zooplankton that emerges from the sea-floor to the water column at night, as suggested by previous biochemical studies at Ningaloo Reef and off the coast of Mozambique (Couturier et al. 2013b, Rohner et al. 2013,

Marcus et al. 2016). Such plankton is an important component of benthic-pelagic coupling in coral reefs as they transport the detrital and bacterial carbon in benthic ecosystems to the surface waters at night (Smith et al. 1979, Alldredge and King, 1985). At Ningaloo Reef for example, whale sharks appear to adapt their diving behavior in response to these nocturnal migrations (Gleiss et al. 2013). Despite demersal zooplankton in this study (e.g. mysids and euphausiids) showing pelagic sources of carbon (δ^{13}C < -17‰, France 1995), emergent copepods were found to be more enriched than pelagic zooplankton in a coastal lagoon (Pitt et al. 2008). These contrasting results are likely due to the fact that zooplankton and nektonic samples collected at Ningaloo were not caught within the reef system, and thus are more dependent on plankton-based food webs from offshore. Overall, the carbon-enriched composition in whale sharks at Ningaloo Reef, thus, supports current evidence of a diet focused on demersal zooplankton inshore.

The whale shark biopsies used in this research consisted of sub-dermal tissue which is likely to integrate long-term dietary information (over months to years) (Marcus et al. 2016) and thus may include isotope signatures on foraging of whale sharks prior to their arrival at Ningaloo. Tagging studies show that these locations include other coastal and shelf areas throughout Western Australia, in addition to occasional travels offshore to the Indian Ocean (Wilson et al. 2006, Sleeman et al. 2010, Norman et al. 2016). In open ocean waters, whale sharks are likely to access food resources from the deep-scattering layer (Rohner et al. 2013, Meekan et al. 2015, Marcus et al. 2016). The relative contribution of deep-water prey to the diet of whale sharks is however, unknown. Future work should focus on more detailed characterization of isotopic signatures of inshore communities and prey in the deep-scattering layer to assist in the interpretation of biochemical analysis. Analysis of tissues with faster turnovers such as blood or plasma might also aid identification of more recent dietary components of whale sharks at Ningaloo Reef. The collection of these types of tissues would however, involve either the restraint of sharks or sampling from free-swimming animals which poses significant ethical and logistic challenges.

4.4.2. Effects of size and sex in diet of whale sharks

Our data suggested an ontogenetic change in the trophic position of whale sharks. In general, δ^{15}N values increased with total length, indicating an increasing contribution of prey from higher trophic levels as whale sharks grow. This shift was more pronounced for females than for males. Ontogenetic shifts in diet are common in elasmobranchs (see Wetherbee and Cortés, 2004), and have also been recorded for whale sharks in the Arabian Sea (Borrell et al. 2011 a,b). Given that neonate whale sharks have rudimentary filtration structures (Garrick

1964), a change in diet with age would be expected as their filter pads develop. This would allow dead-end sieving or cross-flow filtration to commence, enabling whale sharks to feed on larger prey and higher-trophic level prey (Motta et al. 2010), such as small bait fish and macro-zooplankton (Duffy 2010, Rohner et al. 2015). The increase in δ^{15}N values due to dietary shifts represented an increase of 0.76 in the trophic position occupied by whale sharks.

The observed variation in δ^{15}N values might also indicate spatial segregation among whale sharks. For instances, basin-wide gradients in δ^{15}N values > 12‰ or of ~5‰ at a more regional scale, were observed in muscle of tuna within the Pacific Ocean that was consistent with differences in the nitrogen isotopic composition at the base of the food web (Popp et al. 2007, Graham et al. 2010). Although nitrogen isotopic baselines in the eastern Indian Ocean are poorly characterized, recent observations suggest that differences among regions are likely to occur. Notably, low values of δ^{15}N from N_2 fixation and recycling of organic matter are characteristic of food webs in offshore surface waters, whereas more enriched signatures are found in food webs in coastal waters and at depth (Waite et al. 2013, Raes et al. 2014). Consistent with Borrell et al. (2011 a,b), the observed enrichment of ^{15}N signatures with size in both males and females at Ningaloo suggests a transition in habitat from offshore pelagic waters to more inshore benthic environments as whale sharks grow.

Ontogenetic migrations from offshore environments to inshore habitats are consistent with the known ecology of whale sharks. There is evidence to suggest that breeding sites in the Indian Ocean are located far from the coast and that neonate and small (< 3 m TL) whale sharks are then confined to pelagic natal habitats (Rowat et al. 2008). Because of the scarcity of records of small whale sharks at Ningaloo Reef (Norman and Stevens, 2007, Sequeira et al. 2016), migrations from offshore areas to more productive coastal habitats are likely to commence at around 3 m TL. These movements could result in smaller individuals, which might have recently arrived at Ningaloo Reef, still showing evidence of offshore pelagic diets in their isotope signatures. As the aggregation at Ningaloo Reef is dominated by juvenile males, this raises the question as to the location and target prey of both juvenile and adult females. Although a few juvenile females were sampled by this study, no adults were encountered. It seems likely that adult females inhabit deeper offshore waters at Ningaloo, as is the case in the Gulf of California, Belize, Galápagos or the Arabian Sea (Graham and Roberts, 2007, Ramírez-Macías et al. 2017, Borrell et al. 2011 a,b, Ketchum et al. 2013, Hearn et al. 2016). Meekan et al (2015) suggest that for whale sharks, aggregations of juvenile males occur along the coast to take advantage of local increases in prey abundance that can be exploited without the metabolic costs entailed by feeding in deep water in the open ocean (Thums et al. 2015).

For adult sharks, these costs are minimized by their very large size, so that these animals occur primarily in oceanic habitats and for the most part, do not need to feed in coastal habitats (Meekan et al. 2015). However, why juvenile female sharks do not, for the most part, take advantage of the opportunity to feed in coastal waters is not known.

4.5. Conclusions

The present study has shown that whale sharks sampled at Ningaloo Reef have a planktivorous diet associated with inshore benthic food webs. The target species are likely to include members of the demersal zooplankton in these ecosystems. Overall, isotopic compositions indicated a shift in the trophic position, the movement patterns and the feeding behavior of whale sharks according to their size and sex. The feeding habits of individual whale sharks are thus likely to differ depending on the habitat they occupy, ranging from a diet highly reliant on offshore pelagic prey to more inshore benthic food chains as the animals grow (~3 to 8m). Future studies should account for these size- and sex-specific patterns of feeding by broadening sampling programs to include female and adult whale sharks. In a population that is already at risk, whale sharks are commercially exploited either by fisheries and/or by a growing ecotourism industry in many parts of their distribution (Pierce and Norman, 2016). High dependency on coastal habitats, where most of the exploitation activity is concentrated, and spatial differences in foraging patterns by sex and size, suggests that localized impacts could affect whale shark populations at a larger scale (Wearmouth and Sims, 2008, Speed et al. 2012). Management and conservation strategies should therefore be directed to protect key habitats for the species at their different life stages to ensure connectivity for this highly mobile elasmobranch.

Chapter 5

General discussion, recommendations and future research

5.1. General discussion

Whale sharks have recently been categorized as an “Endangered” species (IUCN, 2016, Pierce and Norman, 2016), highlighting the urgent need for an improvement in scientific knowledge of the species for a better implementation of conservation strategies. The research presented in this thesis aims to provide some of this data by describing the feeding ecology of whale sharks. This was achieved through the development of improved biochemical techniques and interpretation of analytical results.

5.1.1. Sub-dermal tissue of whale sharks (Chapter 2 and 4)

Knowledge of the turnover rate or incorporation time of trophic tracers (e.g. fatty acids or stable isotopes of carbon and nitrogen) into tissues is critical to the appropriate assessment of the feeding ecology of an animal (Martínez del Río et al. 2009, Hussey et al. 2012). This requires controlled laboratory studies, which are presently non-existent for sub-dermal tissue of elasmobranchs. However, as observed here, sub-dermal tissue is structural in nature and likely to resemble muscle and cartilage in terms of incorporation rates of biochemical markers, thus providing medium to long-term dietary information over months or even years (MacNeil et al. 2006, Logan and Lutcavage, 2010, Kim et al. 2012). Given that our sampling was undertaken in the middle of the whale shark season in May, dietary information in the present study might include feeding events of whale sharks prior to their arrival at Ningaloo Reef, in addition to time spent at the aggregation site. The use of different tissues with faster turnover rates (days - weeks) such as liver or blood could offer short-term dietary information and in combination with sub-dermal tissue samples could provide a more comprehensive understanding of the foraging behavior of whale sharks. However, difficulties in collecting these samples hamper our ability to obtain this information.

Sub-dermal tissue has potential to be used in FA and SIA studies, especially on those involving high-risk conservation species, as sample collection is the least invasive for the animal. This tissue should therefore be the focus of attention in future laboratory work. Alternatively, other techniques such as the analysis of parasitic copepods should be explored as potential tools to provide recent diet knowledge without the necessity to harm the animal.

5.1.2. Standardized protocols for samples prior to SIA (Chapter 3)

In this study, we proposed protocols for the treatment of elasmobranch tissues and their potential prey to standardize δ^{13}C and δ^{15}N values of samples prior to SIA. This is an essential step for the correct interpretation of SIA data and comparison of results within and among studies. Lipids and urea should be extracted from sub-dermal tissue of whale sharks using LE+DIW treatment. Similar results have been observed in previous studies with other shark species (Kim and Koch, 2012, Carlisle et al. 2016, Li et al. 2016), suggesting that this step should be a requirement with elasmobranch tissues prior to SIA. In addition, our results suggest that the standard approach of using C:N ratios to normalize δ^{13}C values for lipid content in marine animals (Post et al. 2007) may confound some results. In contrast, this study recommends lipid extraction of samples of zooplankton and nektonic taxa for the analysis of carbon. Although results presented here suggest that samples containing inorganic carbonate should be acidified, more experimental work is needed to fully understand the relationship between inorganic carbonate content and effects on δ^{13}C values. We have also confirmed that δ^{15}N values are further altered by lipid and inorganic carbon extraction, thus an untreated aliquot should be analyzed for nitrogen. Finally, in order to avoid extra cost and time, it is possible to develop lipid correction models using a subset of the samples.

5.1.3. Whale shark feeding ecology (Chapter 2 and 4)

Signature FA profiles and stable isotopes have proven to be useful and complementary trophic tracers that can help to answer important ecological questions about the feeding habits of whale sharks at broad temporal and spatial scales. Individually, FA data of whale shark sub-dermal tissue was more sensitive for detecting dietary sources, and in turn variations in diet between years and among individuals (Chapter 2). In contrast, SIA gave estimates of trophic position and better insights into the role of whale sharks within regional food webs, and was a more sensitive indicator for revealing patterns of feeding and habitat usage according to size and sex (Chapter 4).

The size and sex ratio of whale sharks aggregating at Ningaloo Reef was clearly biased towards juvenile males of 3 to 8.5 m TL. This result was expected, since this demographic group appears to be dominant in coastal aggregation sites world-wide including at Ningaloo Reef (Meekan et al. 2006, Rowat et al. 2011, Rowat and Brooks, 2012, Araujo et al. 2017). These whale sharks had a mean estimate trophic position of 2.6, which was lower than previously observed by stomach content or stable isotope analysis in other Indian Ocean aggregations (Cortés 1999, Borrell et al. 2011b). Differences in trophic position between

studies might be due to biased representation of some prey items in stomach contents, the use of different diet-tissue discrimination factors when estimating trophic positions or by the fact that whale sharks at Ningaloo might be feeding on prey with lower values of δ^{15}N within the food web than in other locations. In comparison, whale sharks in this study had trophic roles similar to planktivorous reef fishes at Ningaloo (Wyatt et al. 2012b, 2013).

Both FA signatures and SIA suggested high dependency on inshore benthic food webs by whale sharks in this study, data consistent with biochemical studies of these sharks off the coast of Mozambique (Couturier et al. 2013b, Rohner et al. 2013). These earlier studies, which mainly sampled populations dominated by juvenile males in environments very close to the coast, suggested that demersal or emergent zooplankton were key components of the whale shark diet. Despite our efforts to sample potential prey of whale sharks at Ningaloo, we were not able to directly confirm this hypothesis. Biochemical profiles of collected prey, which were characteristic of pelagic environments, were different to those of whale sharks. However, the high levels of the ω6 LC-PUFA – ARA - coupled with the consistent enrichment in ^{13}C observed in whale shark sub-dermal tissue, demonstrated dietary sources from demersal prey (Copeman and Parrish, 2003, Connelly et al. 2014, Pitt et al. 2008). It is likely that our observations also reflect feeding in coastal and across shelf environments of Western Australia in addition to meso-pelagic waters in the open ocean used by whale sharks prior their arrival to Ningaloo Reef (Wilson et al. 2006, Sleeman et al. 2010, Meekan et al. 2015, Norman et al. 2016). Future work should expand on characterizing the isotopic and FA signatures of benthic and demersal prey from coastal and offshore environments, to assist in the interpretation of biochemical results. Overall, the broad foraging range of whale sharks suggested by this and recent studies at coastal aggregations (Couturier et al. 2013b, Rohner et al. 2013), challenges our current understanding that whale sharks are pelagic filter-feeders targeting prey in surface waters as previously assumed (e.g. Clark and Nelson, 1997, Heyman et al. 2001, Taylor 2007).

Our data indicated high intraspecific variability in the diet of whale sharks aggregating at Ningaloo Reef. Four different groups of whale shark FA profiles were characterised in 2013 and five groups in 2014. As this variation was not related to size or sex, variability in the diet was seen as being potentially linked to the different feeding grounds used by individual or groups of whale sharks. These results reflected the wide range of horizontal and vertical movements shown by whale sharks when travelling both to and from Ningaloo Reef, and while resident at the aggregation (Wilson et al. 2006, Sleeman et al. 2010, Norman et al. 2016). The inter-annual variations in FA profiles of whale sharks observed in this study were likely driven by temporal changes in profiles at the base of the food web (Budge et al. 2008, Pethybdrige

et al. 2015). The same pattern of variation was seen in zooplankton samples collected in 2013 and 2014, which was further evidence of temporal changes in the baseline signatures. Ultimately, intraspecific variability in diet of whale sharks reflected the patchy and variable nature of foraging in oligotrophic tropical waters, where food is sparse in both time and space. Such variation might increase through time as the marine environment changes due to over-exploitation of resources and climate change.

Unlike FA signatures, SIA suggested that the feeding habits of whale sharks vary by size and sex. We found that the isotopic composition of whale sharks at Ningaloo differed to those of larger females sampled by Borrell et al. (2011 a,b) off the coast of India, which showed a diet highly reliant on pelagic zooplankton. Differences in the feeding habits might be related to the different habitats occupied by these whale sharks. Juvenile males preferentially aggregate in coastal habitats to take advantage of localized prey pulses, whereas larger adult whale sharks are often reported offshore (Graham and Roberts, 2007, Borrell et al. 2011 a,b, Ketchum et al. 2013, Ramírez-Macías et al. 2017), where they may reside because of dietary preferences, for breeding, mating or other purposes. Our results, based on an increase in the values of δ^{15}N with size, also indicated ontogenetic shifts in diet and habitat for whale sharks at Ningaloo. As size increased (3 – 8 m), whale sharks moved from offshore pelagic environments to coastal habitats. Therefore, size- and sex-specific foraging differences should be taken into account when investigating and describing the feeding ecology of the species.

Biochemical techniques used in this thesis could be extended to other whale shark aggregations to acquire further ecological knowledge of the species at larger temporal and spatial scales. Future studies should direct efforts to sample a greater range of sizes and a more female whale sharks, which are under-represented in feeding aggregations. Despite the usefulness of biochemical analysis to examine diet and foraging ranges of whale sharks, these approaches may be further enhanced when applied in combination with other techniques including satellite tagging and genetic studies, and stomach content analysis on an opportunistic basis. Relatively newer techniques such as compound-specific stable isotope analysis of fatty acids or amino acids, have the potential to estimate trophic position and migratory movements of highly mobile animals without some of the limitations posed by SIA (Popp et al. 2007). Although still not widely applied, these techniques are becoming more popular.

5.2. Concluding remarks

As shown in this study, the feeding ecology of whale sharks in the Eastern Indian Ocean, and possibly those of other populations world-wide, is complex. Juvenile males, the dominant demographic group at Ningaloo Reef, show high reliance on coastal environments and are likely to feed on demersal zooplankton rather than on pelagic prey. The absence of larger or female whale sharks at Ningaloo, a result that is consistent with other coastal aggregation sites, suggest that adults occupy other habitats, and that they might display different feeding habits. High intraspecific variability in the feeding ecology of whale sharks shown in this study is likely a reflection of the dynamics of the tropical environment where food is sparse in time and space, as well as the intrinsic biological traits of the species.

Human exploitation of segregated groups of sharks (sex and/or size) may be of particular concern to population stability. The preferential use of coastal habitats by juvenile males, highlights the issue that this age group could be particularly vulnerable to commercial exploitation in part of their range. Such harvesting could threaten the viability of the sub-population in the Eastern Indian Ocean, and the related ecotourism industry. Thus, further investigation of whale shark movement patterns including migratory pathways, habitat use, and feeding grounds at their different life stages should be key research priorities. With a more in depth understanding, both global and state management and conservation bodies will be able to protect critical habitats for the species.

References

Abrantes KG, Barnett A (2011) Intrapopulation variations in diet and habitat use in a marine apex predator, the broadnose sevengill shark *Notorynchus cepedianus*. Mar Ecol Progr Ser 442:133-148

Abrantes KG, Semmens JM, Lyle JM, Nichols PD (2011) Normalisation models for accounting for fat content in stable isotope measurements in salmonid muscle tissue. Mar Biol 159: 57-64

Albo-Puigserver M, Navarro J, Coll M, Aguzzi J, Cardona L, Sáez-Liante R (2015) Feeding ecology and trophic position of three sympatric demersal chondricthyans in the northwestern Mediterranean. Mar Ecol Prog Ser 524:255-268

Alldregde AL, King JM (1985) The distance demersal zooplankton migrate above the benthos: implications for predation. Mar Biol 84:253-260

Andrzejaczek S, Meeuwig J, Rowat D, Pierce S, Davies T, Fisher R, Meekan M (2016) The ecological connectivity of whale shark aggregations in the Indian Ocean: a photo-identification approach. R Soc Open Sci 3:160455

Araujo G, Vivier F, Labaja JJ, Hartley D, Ponzo A (2017) Assessing the impacts of tourism on the world's largest fish *Rhincodon typus* at Panon Island, Southern Leyte, Phillipines. Aquatic Conserv: Mar Freshw Ecosyst 1-9

Barton K (2013) Package 'MuMIn': Multi-model inference. 43

Beckmann CL, Mitchell JG, Stone DAJ, Huveneers C (2014) Inter-tissue differences in fatty acid incorporation as a result of oil manipulation in Port Jackson sharks (*Heterodontus portysjacksoni*). Lipids 49:577-590

Beckmann CL, Mitchell JG, Seuront L, Stone DAJ, Huveneers C (2013) Experimental evaluation of fatty acid profiles as a technique to determine dietary composition in benthic elasmobranchs. Physiol Biochem Zool 86(2):266-278

Best NJ, Bradshaw CJ, Hindell MA, Nichols PD (2003) Vertical stratification in fatty acids in the blubber of southern elephant seals (*Mirounga leonine*): implications for diet analysis. Comp Biochem Phys 134:253-263

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917

Bodin N, Le Loc'h F, Hily C (2007) Effect of lipid removal on carbon and nitrogen stable isotope ratios in crustacean tissues. J Exp Mar Biol Ecol 341:168-175

Borrell A, Aguilar A, Gazo M, Kumarran RP, Cardona L (2011a) Stable isotope profiles in whale shark (*Rhincodon typus*) suggest segregation and dissimilarities in the diet depending on sex and size. Environ Biol Fish 92:559-567

Borrell A, Cardona L, Kumarran RP, Aguilar A (2011b) trophic ecology of elasmobranchs caught off Gujarat, India, as inferred from stable isotopes. ICES J Mar Sci 68(3):547-554

Bosley KL, Wainright SC (1999) Effects of preservatives and acidification on the stable isotope ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. Can J Fish Aquat Sci 56: 2181-2185

Bowen WD, Iverson SJ (2013) Methods of estimating marine mammal diets: a review of validation experiments and sources of bias and uncertainty. Mar Mammal Sci 29(4):719-754

Bradshaw CJA, Fitzpatrick BM, Steinberg CC, Brook BW, Meekan MG (2008) Decline in whale sharks size and abundance at Ningaloo Reef over the past decade: the world's largest fish is getting smaller. Biol Conserv 141(7):1894-1905

Bradshaw CJA, Hindell MA, Best NJ, Phillips KL, Wilson G, Nichols PD (2003) You are what you eat: describing the foraging ecology of southern elephant seals (*Mirounga leonine*) using blubber fatty acids. Proc R Soc Lond B 270:1283-1292

Breheny P, Burchett W (2016) Package 'visreg' : Visualization of Regression Models. 14

Brunnschweiler JM, Sims DW (2011) Diel Oscillations in whale shark vertical movements associated with meso- and bathypelagic diving. Am Fish Soc Symp 76:1-13

Budge SM, Springer AM, Iverson SJ, Sheffield G, Rosa C (2008) Blubber fatty acid composition of bowhead whales, *Balaena mysticetus*: Implications for diet assessment and ecosystem monitoring. J Exp Mar Biol Ecol 359:40-46

Budge SM, Iverson SJ, Koopman HN (2006) Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. Mar Mamm Sci 22: 759-801

Bunn SE, Loneragan NR, Kempster MA (1995) Effects of acid washing on stable isotope ratios of C and N in penaeid shrimp and seagrass: implications for food-web studies using multiple stable isotopes. Limnol Oceanogr 40(3):622-625

Burgess KB, Bennett M B (2016) Effects of ethanol storage and lipid and urea extraction on δ^{15}N and δ^{13}C isotope ratios in a benthic elasmobranch, the bluespotted maskray *Neotrygon kuhlii*. J Fish Biol 90:417-423

Burnham KP, Anderson DR (2002) Model Selection and multimodel inference: A practical information-theoretic approach. Springer, New York

Carabel S, Godínez-Domínguez E, Verísimo P, Fernández L, Freire J (2006) An assessment of sample processing methods for stable isotope analyses of marine food webs. J Exp Mar Biol Ecol 336:254-261

Carlisle AB, Litvin SY, Madigan DJ, Lyons K, Bigman JS, Ibarra M, Bizzarro JJ (2016) Interactive effects of urea and lipid content confound stable isotope analysis in elasmobranch fishes. Can J Fish Aquat Sci dx.doi.org/10.1139/cjfas-2015-0584

Cassata L, Collins LB (2008) Coral reef communities, habitats, and substrates in and near sanctuary zones of Ningaloo Marine Park. J Coast Res 24:139-151

Catlin J, Jones T, Norman B, Wood D (2010) Consolidation in a wildlife tourism industry: the changing impact of whale shark tourist expenditure in the Ningaloo Reef Coast Region. Inter J Tourism Res 12:134-148

Churchill DA, Heithaus MR, Grubbs RD (2015) Effects of lipid and urea extraction on δ^{15}N values of deep-sea sharks and hagfish: Can mathematical correction factors be generated? Deep-Sea Res II 115:103-108

Clark E, Nelson DR (1997) Young whale sharks, *Rhincodon typus*, feeding on a copepod bloom near La Paz, Mexico. Env Biol Fish 50:63-73

Colman JG (1997) A review of the biology and ecology of the whale shark. J Fish Biol 51:1219-1234

Compagno LJV (2001) Sharks of the World: An annotated and illustrated catalogue of shark species known to date. Vol 2. Bullhead, mackerel and carpet sharks (Heterodontiformes, Lamniformes and Orectolobiformes). FAO Spec. Cat. Fish. Purp. 1(2):269

Connelly TL, Deibel D, Parrish CC (2014) Trophic interactions in the benthic boundary layer of the Beaufort Sea shelf, Arctic Ocean: combining bulk stable isotope and fatty acid signatures. Prog Ocenogr 120:79-92

Copeman LA, Parrish CC (2003) Marine lipids in a cold coastal ecosystem: Gilbert Bay, Labrador 143:1213-1227

Cortés E (1999) Standardized diet compositions and trophic levels of sharks. ICES J Mar Sci 56:707-717

Couturier LIE, Rohner CA, Richardson AJ, Marshall AD, Jaine FRA, Bennett MB, Townsend KA, Weeks SJ, Nichols PD (2013a) Stable isotope and signature fatty acids analyses suggest reef manta rays feed on demersal zooplankton. PLoS ONE 10:e77152

Couturier LIE, Rohner CA, Richardson AJ, Pierce SJ, Marshall AD, Jaine FRA, Townsend KA, Bennett MB, Weeks SJ, Nichols PD (2013b) Unusually high levels of ω6 polyunsaturated fatty acids in whale sharks and reef manta rays. Lipids 48:1029-1034

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003) Fatty acid trophic markers in the pelagic marine environment. Adv Mar Biol 46:225-340

Davidson BC, Nel W, Rais A, Namdarizandi V, Vizarra S, Cliff G (2014) Comparison of total lipids and fatty acids from liver, heart and abdominal muscle of scalloped (*Shpyrna lewini*) and smooth (*Sphyrna zygaena*) hammerhead sharks. SpringerPlus 3:521

DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. Geochim Cosmochim 45:341-345

DeNiro MJ, Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. Geochim Cosmochim 42:49-506

DeNiro MJ, Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197:261-263

Deudero S, Pinnegar JK, Polunin VC (2002) Insights into the fish host-parasite trophic relationships revealed by stable isotope analysis. Dis Aquat Organ 52:77-86

Duffy CAJ (2010) Distribution, seasonality, lengths, and feeding behavior of whale sharks (*Rhincodon typus)* observed in New Zealand waters. New Zea J Mar Fresh Res 36:3

Ebert DA, Fowler S, Compagno L (2013) Sharks of the world. A fully illustrated guide. Wild Nature Press, Plymouth

Fisk AT, Tittlemier SA, Pranschke JL, Norstrom RJ (2002) Using anthropogenic contaminants and stable isotopes to assess the feeding ecology of Greenland sharks. Ecology 83(8):2162-2172

France R (1995) Carbon-13 enrichment in benthic compared to planktonic algae: food web implications. Mar Ecol Prog Ser 124:307-312

François R, Altabet MA, Goericke R, McCorkle DC, Brunet C, Poisson A (1993) Changes in the δ^{13}C of surface water particulate organic matter across the subtropical convergence in the SW Indian Ocean. Glob Biogeochem Cycl 7(3):627-644

Garrick JAF (1964) Additional information on the morphology of an embryo whale shark. Proc US Nat Mus 115:10

Gleiss A, Wright S, Liebsch N, Wilson RP, Norman B (2013) Contrasting diel patterns in vertical movement and locomotor activity of whale sharks at Ningaloo Reef. Mar Biol 160:2981-2991

Goericke R, Fry B (1994) Variations of marine phytoplankton δ^{13}C with latitude, temperature, and dissolved CO_2 in the world ocean. Glob Biogeochem Cycl 8(1):85-90

Goering J, Alexander V, Haubenstock N (1990) Seasonal variability of stable carbon and nitrogen isotope ratios of organisms in a North Pacific bay. Estuar Coast Shelf S 30:239-260

Graham BS, Koch PL, Newsome SD, McMahon KW, Aurioles D (2010) Using isoscapes to trace the movements and foraging behavior of top predators in oceans ecosystems. In: West JB (eds) Isoscapes: Understanding movement, pattern and process on earth through isotope mapping. Springer

Graham R, Roberts CM (2007). Assessing the size, growth rate and structure of a seasonal population of whale sharks (*Rhincodon typus* Smith 1828) using conventional tagging and photo identification. Fisheries Research 84:71–80

Graham RT, Roberts CM, Smart JCR (2006) Diving behaviour of whale sharks in relation to a predictable food pulse. J R Soc Interface 3:109-116

Hearn AR, Green J, Román MH, Acuña-Marrero D, Espinoza E (2016) Adult female whale sharks make-long-distance movements past Darwin Island (Galapagos, Ecuador) in the Eastern Tropical Pacific. Mar Biol 163:214

Heyman WD, Graham RT, Kjerfve B, Johannes RE (2001) Whale shark *Rhincodon typus* aggregate to feed on fish spawn in Belize. Mar Ecol Prog Ser 215:275-282

Hobson KA, Barnett-Johnson R, Cerling T (2010) Using isoscapes to track animal migration. In: West JB (eds) Isoscapes: Understanding movement, pattern and process on earth through isotope mapping. Springer

Hobson KA, Sease JL, Merrick RL, Piatt JF (1997) Investigating trophic relationships of pinnipeds in alaska and Washington using stable isotope ratios of nitrogen and carbon. Mar Mammal Sci 13(1):114-132

Hueter RE, Tyminski JP, de la Parra R (2013) Horizontal movements, migration patterns, and population structure of whale sharks in the Gulf of Mexico and Northwestern Caribbean Sea. PLoS ONE 8:e71883

Hussey NE, MacNeil MA, McMeans BC, Olin JA, Dudley SFJ, Cliff G, Wintner SP, Fennessy ST, Fisk AT (2014) Rescaling the trophic structure of marine food webs. Ecol Lett 17:239-250

Hussey NE, Olin JA, Kinney MJ, McMeans BC, Fisk AT (2012) Lipid extraction effects on stable isotope values (δ^{13}C and δ^{15}N values) of elasmobranch muscle tissue. J Exp Mar Biol Ecol 434-435: 7-15
Ihaka R, Gentleman R (1996) A language for data analysis and graphics. J Comput Graph Stat 5:299-314

Hussey NE, Chapman DD, Donnelly E, Abrcrombie DL, Fisk AT (2011) Fin-icky samples: as assessment of shark fin as source material for stable isotope analysis. Limnol Oceanogr 9:524-532

Hussey NE, Brush J, McCarthy I, Fisk AT (2010) δ^{13}C and δ^{15}N diet-tissue discrimination factors for large sharks under semi-controlled conditions. Comp Biochem Physiol A Mol Integr Physiol 155:445-453

Ingram T, Matthews B, Harrod C, Stephens T, Grey J, Markel R, Mazumder A (2007) Lipid extraction has little effect on the δ^{15}N of aquatic consumers. Limnol Oceanogr-Meth 5:338-343

Integrated Marine Observing System (2013, 2014), IMOS Ocean Current: Surface Currents and Temperatures http://oceancurrent.imos.org.au/index.php. (accessed 22nd May 2015)

Iverson SJ (2009) Tracing aquatic food webs using fatty acids: from qualitative indicators to quantitative determination. In: Arts MT, Brett MT, Kainz MJ (eds) Lipids in aquatic ecosystems. Springer, New York, NY, p 281-308

Iverson SJ, Springer AM, Kitaysky AS (2007). Seabirds as indicators of food web structure and ecosystem variability: qualitative and quantitative diet analyses using fatty acids. Mar Ecol Prog Ser 352:235-244

Ivlev A, Knyazev YA, Logachev M (1996) Daily average carbon isotope composition of CO_2 of expired air and urine in norm and some endocrine pathologies in man. Biofizika 41(2):508-516

Jacob U, Mitenbeck K, Brey T, Knust R, Beyer K (2005) Stable isotope food web studies: a case for standardized sample treatment. Mar Ecol Prog Ser 287:251-253

Jarman SN, Wilson SG (2004) DNA-based species identification of krill consumed by whale sharks. J Fish Biol 65:586-591

Jaschinski S, Hansen T, Sommer U (2008) Effects of acidification in multiple stable isotope analyses. Limnol Oceanogr: Methods 6:12-15

Jayasinghe C, Gotoh N, Tokairin S, Ehara H, Wada S (2003) Inter species changes of lipid composition in liver of shallow-water sharks from the Indian Ocean. Fish Sci 69:644-653

Johannsson OE, Leggett MF, Rudstam LG, Servos MR, Mohammadian MA, Gal G, Dermott RM, Hesslein RH (2011) Diet of *Mysis relicta* in Lake Ontario as revealed by stable isotope and gut content analysis. Can J Fish Aquat Sci 58: 1975-1986

Johns RB, Nichols PD, Perry GJ (1979) Fatty acid composition of ten marine algae from Australian waters. Phytochemistry 18:799-802

Ketchum JT, Galván-Magaña F, Klimley AP (2013) Segregation and foraging ecology of whale sharks, *Rhincodon typus*, in the southern western Gulf of California. Environ Biol Fish 96:779-795

Kiljunen M, Grey J, Sinisalo T, Harrod C, Immonen H, Jones RI (2006) A revised model for lipid-normalizing δ^{13}C values from aquatic organisms, with implications for isotope mixing models. J Appl Ecol 43(6):1213-1222

Kim SL, Koch PL (2012) Methods to collect, preserve, and prepare elasmobranch tissues for stable isotope analysis. Environ Biol Fish 95:53-63

Kim SL, Martínez del Rio C, Casper D, Koch PL (2012) Isotopic incorporation rates of shark tissues from a long-term captive feeding study. J Exp Biol 215:2495-2500

King P, Kennedy H, Newton PP, Jickells TD, Brand T, Calvert S, Cauwet G, Etcheber H, Head B, Khripounoff A, Manighetti B, Miquel JC (1998) Analysis of total and organic carbon and total nitrogen in settling oceanic particles and a marine sediment: an inter-laboratory comparison, Mar Chem 60:203-216

Lee Chang KJ, Dunstan GA, Abell GCJ, Clemenston LA, Blackburn SI, Nichols PD, Koutoulis A (2012) Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils. Appl Microbiol Biotechnol 93:2215-2231

Li Y, Zhang Y, Hussey NE, Dai X (2016) Urea and lipid extraction treatment effects on δ^{15}N and δ^{13}C values in pelagic sharks. Rapid Commun Mass Spectrom 30:1-8

Logan JM, Lutcavage ME (2010) Stable isotope dynamics in elasmobranch fishes. Hydrobiologia 644:231-244

Logan JM, Jardine TD, Miller TJ, Bunn SE, Cunjak RA, Lutcavage ME (2008) Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modelling methods. J Anim Ecol 77:838-846

MacNeil MA, Drouillard KG, Fisk AT (2006) Variable uptake and elimination of stable nitrogen isotopes between tissues in fish. Can J Fish Aquat Sci 63:345-353

Marcus L, Virtue P, Nichols PD, Meekan MG, Pethybridge HP (2017) Effects of sample treatment on the analysis of stable isotopes of carbon and nitrogen in zooplankton, micronekton and a filter-feeding shark. Mar Biol 164:124

Marcus L, Virtue P, Pethybridge HR, Meekan MG, Thums M, Nichols PD (2016) Intraspecific variability in diet and implied foraging ranges of whale sharks at Ningaloo Reef, Western Australia, from signature fatty acids. Mar Ecol Prog Ser 554:115-128

Martínez del Río C, Wolf N, Carleton SA, Gannes LZ (2009) Isotopic ecology ten years after a call for more laboratory experiments. Biol Rev 84:91-111

Matich P, Heithaus MR, Layman CA (2011) Contrasting patterns of individual specialization and trophic coupling in two marine apex predators. J Anim Ecol 80:294-305

McClelland JW, Montoya JP (2002) Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. Ecology 83:2173-2180

McConnaughey T, McRoy CP (1979) Food-web structure and the fractionation of carbon isotopes in the Bering Sea. Mar Biol 53:257-262

McMeans BC, Arts MT, Fisk AT (2013) Similarity between predator and prey fatty acid profiles is tissue dependent in Greenland sharks (*Somniosus microcephalus*): implications for diet reconstruction. J Exp Mar Biol Ecol 429:55-63

Meekan MG, Fuiman LA, Davis R, Berger Y, Thums M (2015) Swimming strategy and body plan of the world's largest fish: implications for foraging efficiency and thermoregulation. Front Mar Sci 2:64

Meekan MG, Jarman SN, McLean C, Schultz MB (2009) DNA evidence of whale sharks (*Rhincodon typus*) feeding on red crab (*Gecarcoidea natalis*) larvae at Christmas Island, Australia. Mar Freshw Res 60:607-609

Meekan MG, Bardshaw CJA, Press M, McLean C, Richards A (2006) Population size and structure of whale sharks *Rhincodon typus* at Ningaloo Reef, Western Australia. Mar Ecol Prog Ser 319:275-285

Meekan MG, Wilson SG, Halford A, Retzel A (2001) A comparison of catches of fishes and invertebrates by two light trap designs, in tropical NW Australia. Mar Biol 139:373-381

Mitenbeck K, Brey T, Jacob U, Knust R, Struck U (2008) How to account for the lipid effect on carbon stable-isotope ratio (δ^{13}C): sample treatment effects and model bias. J Fish Biol 72:815-830

Monroig O, Tocher DR, Navarro JC (2013) Biosynthesis of polyunsaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms. Mar Drugs 11:3998-4018

Montoya JP (2007) Natural abundance of ^{15}N in marine planktonic ecosystems. In: Michener R, Latjha K (eds) Stable isotopes in ecology and environmental science. Blackwell Publishing Ltd, Oxford, UK

Motta PJ, Maslanka M, Heuter RE, Davis RL, de la Parra R, Mulvany SL, Habberger ML, Stroher JA, Mara KR, Gardiner JM, Tyminski JP, Zeigler LD (2010) Feeding anatomy, filter-feeder rate, and diet of whale sharks *Rhincodon typus* during surface ram filter feeding off the Yucatan Peninsula, Mexico. Zoo 113:199-212

Murry BA, Farrell JM, Teece MA, Smyntek PM (2006) Effect of lipid extraction on the interpretation of fish community trophic relationships determined by stable carbon and nitrogen isotopes. Can J Fish Aquat Sci 63:2167-2172

Nelson JD, Eckert SA (2007) Foraging ecology of whale sharks (*Rhincodon typus*) within Bahía de los Angeles, Baja California Norte, México. Fish Res 84:47-64

Newsome SD, Clementz MT, Koch PL (2010) Using stable isotope biogeochemistry to study marine mammal ecology. Mar Mammal Sci 26(3):509-572

Nichols DS (2003) Prokaryotes and the input of polyunsaturated fatty acids to the marine food web. Microbiol Letters 219:1-7

Nichols P, Rayner M, Stevens J (2001) A pilot investigation of northern australian shark liver oils: characterization and value-adding. FRDC Project Report 99/369. CSIRO Marine Research and Fisheries Research and Development Corporation, Australia

Norman BM, Reynolds S, Morgan DL (2016) Does the whale shark aggregate along the Western Australian coastline beyond Ningaloo Reef? Pac Conserv Biol 22:72-80

Norman BM, Stevens JD (2007) Size and maturity status of the whale shark (*Rhincodon typus*) at Ningaloo Reef in Western Australia. Fish Res 84:81-86

Olson KR 1(999) Rectal gland and volume homeostasis, in: Hamlett, W.C. (Eds.), Sharks, skates and rays: the biology of elasmobranchs fishes. The John Hopkins University Press Baltimore, Maryland, pp. 329-352

Papastamatiou YP, Friedlander AM, Caselle JE, Lowe CG (2010) Long-term movement patterns and trophic ecology of blacktip reef sharks (*Carcharinus melanopterus*) at Palmyra Atoll. J Exp Mar Biol Ecol 386:92-102

Parrish CC (2013) Lipids in marine ecosystems. Oceanograh 2013:604045

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Ann Rev Ecol Syst 18:293-320

Pethybridge, H. R., Parrish, C. C., Morrongiello, J., Young, J. W., Farley, J. H., Gunasekera, R. M., & Nichols, P. D. (2015). Spatial Patterns and Temperature Predictions of Tuna Fatty Acids: Tracing Essential Nutrients and Changes in Primary Producers. *PloS one*, *10*(7), e0131598

Pethybridge HR, Parrish CC, Bruce BD, Young JW, Nichols PD (2014) Lipid, fatty acid and energy density profiles of white sharks: insights into the feeding ecology and ecophysiology of a complex top predator. PLoS ONE 5:e97877

Pethybridge H, Daley RK, Nichols PD (2011) Diet of demersal sharks and chimaeras inferred by fatty acid profiles and stomach content analysis. J Exp Mar Biol Ecol 409:290-299

Pethybridge H, Daley R, Virtue P, Nichols P (2010) Lipid composition and partitioning of deepwater chondrichthtans: inferences of feeding ecology and distribution. Mar Biol 157:1367-1384

Pierce SJ, Norman B (2016) *Rhincodon typus*. The IUCN Red List of Threatened Species 2016: e.T19488A2365291

Pinnegar JK, Polunin NVC (1999) Differential fractionation of δ^{13}C and δ^{15}N among fish tissues: implications for the study of trophic interactions. Funct Ecol 13:225-231

Pitt KA, Clement AN, Connolly RM, Thibault-Botha D (2008) Predation by jellyfish on large and emergent zooplankton: implications for benthic-pelagic coupling. Estuar, Coast Shelf Sci 76:827-833

Pomerleau C, Winkler G, Sastri A, Nelson RJ, Williams WJ (2014) The effect of acidification and the combined effects of acidification/lipid extraction on carbon stable isotope ratios for sub-arctic and arctic marine zooplankton studies. Polar Biol 37:1541-1548

Popp BN, Graham BS, Olson RJ, Hannides CCS, Lott MJ, López-Ibarra GA, Galván-Magaña F, Fry B (2007) Insight into the trophic ecology of yellowfin tuna, Thunnus albacares, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. In: Dawson T, R. Siegwolf (eds) Stable isotopes as indicators of ecological change. Elsevier Academic Press, San Diego, CA

Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J, Montaña CG (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analysis. Oecologia 152:179-189

Post DM (2002) Using stable isotopes to estimate trophic position: models, methods and assumptions. Ecology 83(3):703-718

Potvin J, Goldbogen JA, Shadwick RE (2012) Metabolic expenditures of lunge feeding rorquals across scale: implications for the evolution of filter feeding and the limits to maximum body size. PLoS ONE 7(9):e44854

Raes EJ, Waite AM, McInnes AS, Olsen H, Nguyen HM, Hardman-Mountford N, Thompson PA (2014) Changes in latitude and dominant diazotrophic community alter N_2 fixation. Mar Ecol Progr Ser 516:85-102

Ramírez-Macías D, Quieroz N, Pierce SJ, Humphries NE, Sims DW, Brunnschwiler JM (2017) Oceanic adults, coastal juveniles: tracking the habitat use of whale sharks off the Pacific coast of Mexico. PeerJ 5:e3271

Remme JF, Larssen WE, Bruheim I, Saebo PC, Saebo A, Stokens IS (2006) Lipid content and fatty acid distribution in tissues from Portuguese dogish, leafscale gulper shark and black dogfish. Comp Biochem Physiol B 143:459-464

Robinson DP, Jaidah MY, Jabado RW, Lee-Brooks K, Nour El-Din NM, Al Malki AA, Elmeer K, McCormick PA, Henderson AC, Pierce SJ, Ormond RFG (2013) Whale shark, *Rhincodon typus*, aggregate around offshore platforms in Qatari waters of the Arabian Gulf to feed on fish spawn. PLoS ONE 3:e58255

Rohner CA, Armstrong AJ, Pierce SJ, Prebble CEM, Cagua EF, Cochran JEM, Berumen ML, Richardson AJ (2015) Whale sharks target dense prey patches of sergestids shrimp off Tanzania. J Plankton Res 37(2):352-362

Rohner CA, Couturier LIE, Richardson AJ, Pierce SJ, Prebble CEM, Gibbons MJ, Nichols PD (2013) Diet of whale sharks *Rhincodon typus* inferred from stomach content and signature fatty acid analyses. Mar Ecol Prog Ser 493:219-235

Rowat D, Brooks KS (2012) A review of the biology, fisheries and conservation of the whale shark *Rhincodon typus.* J Fish Biol 80:1019-1056

Rowat D, Brooks K, March A, McCarten C, Jouannet D, Riley L, Jeffreys G, Perri M, Vely M, Pardigon B (2011) Long-term membership of whale shark (*Rhincodon typus*) in coastal aggregations in Seychelles and Djibouti. Mar Freshw Res (62:621-627

Rowat D, Gore MA, Baloch BB, Islam Z, Ahmad E, Ali QM, Culloch RM, Hameed S, Hasnain SA, Hussain B, Kiani S, Siddiqui J, Ormond RF, Henn N, Khan M (2008) New records of neonatal and juvenile whale sharks (*Rhincodon typus*) from the Indian Ocean. Environ Biol Fish 82:215-219

Ruiz-Cooley RI, Garcia KY, Hetherington ED (2011) Effects of lipid removal and preservatives on carbon and nitrogen stable isotope ratios of squid tissue: implications for ecological studies. J Exp Mar Biol Ecol 407(1):101-107

Sargent JR, Gatten RR, Mcintosh R (1973) The distribution of neutral lipids in shark tissues. J Mar Biol Ass 53:649-656

Schmidt-Nielsen K (1997) Animal physiology: adaptation and environment (5^{th} ed.), Cambridge Univeristy Press, New York

Sequeira AM, Thums M, Brooks K, Meekan MG (2016) Error and bias in size estimates of whale sharks: implications for understanding demography. R Soc Open Sci 3: 150668

Sheridan MA (1988) Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. Comp Biochem Physiol B 90:679-690

Silas EG, Rajagopalan MS (1963) On a recent capture of a whale shark (*Rhincodon typus* Smith) at Tuticorin, with a note on information to be obtained on whale sharks from Indian waters. J Mar Biol Assoc India 5:153-157

Simpfendorfer CA, Heupel MR, White WT, Dulvy NK (2011) the importance of research and public opinion to conservation management of sharks and rays: a synthesis. Mar Fresh Res 62:518-527

Sims DW, Quayle VA (1998) Selective foraging behavior of basking sharks on zooplankton in a small-scale front. Nature 393:460-464

Sleeman JC, Meekan MG, Wilson SG, Polovina JJ, Stevens JD, Boggs GS, Bradshaw CJA (2010) To go or not to go with the flow: environmental influences on whale shark movement patterns. J Exp Mar Biol Ecol 390:84-98

Sleeman JC, Meekan MG, Wilson SG, Jenner CKS, Jenner MN, Boggs GS, Steinberg CC, Bradshaw CJA (2007) Biophysical correlates of relative abundances of marine megafauna at Ningaloo Reef, Western Australia. Mar Fresh Res 58:608-623

Smith DF, Bulleid NC, Campbell R, Higgins HW, Rowe F, Tranter DJ, Tranter H (1979) Marine food-web analysis: an experimental study of demersal zooplankton using isotopically labelled prey species. Mar Biol 54:49-49

Sotiropoulos MA, Tonn WM, Wassenaar LI (2004) Effects of lipid extraction on stable carbon and nitrogen analyses of fish tissue: potential consequences for food web studies. Ecol Freshw Fish 13:155-160

Speed CW, Meekan MG, Field IC, McMahon CR, Abrantes K, Bradshaw CJA (2012) Trophic ecology of reef sharks determined using stable isotopes and telemetry. Coral Reefs 31:357-367

Speed CW, Meekan MG, Russell BC, Bradshaw CJA (2009) Recent whale shark (*Rhincodon typus*) beach strandings in Australia. Mar Bio Rec 2:e15

Sweeting CJ, Polunin NVC, Jennings S (2006) Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratios of fish tissue. Rapid Commun Mass Spestrom 20:595-601

Syväranta J, Rautio M (2010) Zooplankton, lipids and stable isotopes: importance of seasonal, latitudinal and taxonomic differences. Can J Fish Aquat Sci 67:1721-1729

Taylor JG (2007) Ram filter-feeding and nocturnal feeding of whale sharks (*Rhincodon typus*) at Ningaloo Reef, Western Australia. Fish Res 84:65-70

Thums M, Meekan M, Stevens J, Wilson S, Polovina J (2015) Evidence for behavioural thermoregulation by the world's largest fish. J R Soc Interface 20120477

Van Duyl FC, Moodley L, Nieuwland, van Ijzerloo Lenmart, van Soest RWM, Houtekamer M, Meesters EH, Middelburg JJ (2011) Coral cavity sponges depend on reef-derived food resources: stable isotope and fatty acid constraints. Mar Biol 158:1653-1666

Vignaud TM, Maynard JA, Leblois R, Meekan MG, Vázquez-Juárez R, Ramírez-Macías D, Pierce SJ, Rowat D, Berumen ML, Beeravolu C, Baksay S, Planes S (2014) Genetic structure of populations of whale sharks among ocean basins and evidence for their historic rise and recent decline. Mol Ecol 23:2590-2601

Virtue P, Nichols PD (1994) Lipids from the bull kelp *Durvillaea potatorum.* Phytochmeistry 37(3):673-676

Wada E, Mizutani H, Mingawa M (1991) The use of stable isotopes for food web analysis. Crit Rev Food Sci Nutr 30:361-371

Wai TC, Yeung JW, Lam VYY, Leung KMY, Dudgeon D, Williams GA (2012) Monsoons and habitat influence trophic pathways and the importance of terrestrial-marine linkages for estuary sharks. Ecosphere 3(1):8

Waite AM, Roughan M, Tilbrook B, Thompson PA, Feng M, Wyatt ASJ, Raes EJ (2013) Formation and maintenance of high-nitrate, low ph layers in the eastern Indian Ocean and the role of nitrogen fixation. Biogeosci 10:5691-5702

Wearmouth VJ, Sims D (2008) Sexual segregation in marine fish, reptiles, birds and mammals: behaviou patterns, mechanisms and conservation implications. Adv Mar Biol 54:107-170
Werth A (2000) Feeding in marine mammals. In: Swenck K (eds) Feeding. Academic Press, San Diego

Wetherbee BM, Cortés E (2004) Food consumption and feeding habits. In: Carrier JC, Musick JA, Heithaus MR (eds) Biology of sharks and their relatives. CRC Press, Boca Raton, FL

Wilson SG, Polovina JJ, Stewart BS, Meekan MG (2006) Movements of whale sharks (*Rhincodon typus*) tagged at Ningaloo Reef, Western Australia. Mar Biol 148:1157-1166

Wilson SG, Meekan MG, Carleton J, Stewart T, Knott B (2003) Distribution, abundance and reproductive biology of *Pseudeuphausia latifrons* and other euphuasiids on the southern North West Shelf, Western Australia. Mar Biol 42:369-379

Wilson SG, Pauly T, Meekan MG (2001a) Daytime surface swarming by *Pseudeuphausia latifrons* (Crustacea, Euphausiacea) off Ningaloo Reef, Western Australia. Bull Mar Sci 68(1):157-162

Wilson SG, Taylor JG, Pearce AF (2001b) The seasonal aggregation of whale sharks at Ningaloo Reef, Western Australia: currents, migrations and the El Niño/Southern Oscillation. Environ Biol Fish 61:1-11

Wolf N, Carleton SA, Martínez del Río C (2009) Ten years of experimental animal isotopic ecology. Funct Ecol 23:17-16

Wyatt ASJ, Lowe RJ, Humphries S, Waite AM (2013) Particulate nutrient fluxes over a fringing coral reef: source-sink dynamics inferred from carbon to nitrogen ratios and stable isotopes. Limnol Oceanogr 58 (1):409-427

Wyatt ASJ, Falter JL, Lowe RJ, Humphries S, Waite AM (2012a) Oceanographic forcing of nutrient uptake and release over a fringing coral reef. Limnol Oceanogr 57(2):401-419

Wyatt ASJ, Wake AM, Humphries S (2012b) Stable isotope analysis reveals community-level variation in fish trophodynamics across a fringing coral reef. Coral Reefs 31:1029-1044

Yurkowski DJ, Ferguson S, Choy ES, Loseto LL, Brown TM, Muir DCG, Semeniuk CAD, Fisk AT (2016) Latitudinal variation in ecological opportunity and intraspecific competition indicates differences in niche variability and diet specialization of Arctic marine predators. Ecol Evol 6(6):1666-1678

Zybailov B, Coleman MK, Florens L, Washburn MP (2005) Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labelling. Anal Chem 77:6218-6224

Appendix

Appendix 2.1. The mean fatty acid composition (% of total FA ± standard error) of the outer and inner layer of whale shark biopsies (n = 5) collected at Ningaloo Reef in May 2014.

Fatty acid	Outer layer (n = 5)			Inner layer (n = 5)		
14:0	0.2	±	0.1	0.1	±	0.0
15:0	0.2	±	0.0	0.1	±	0.0
16:0	10.0	±	1.2	7.4	±	1.8
i17:0	1.8	±	0.4	1.7	±	0.9
17:0	1.3	±	0.1	0.9	±	0.2
i18:0	0.8	±	0.1	0.6	±	0.1
18:0	18.5	±	0.8	18.3	±	3.6
20:0	0.4	±	0.1	0.5	±	0.1
22:0	0.6	±	0.1	0.4	±	0.1
24:0	0.4	±	0.2	0.4	±	0.2
Total SFA	**34.4**	±	**1.6**	**30.4**	±	**6.4**
16:1ω9c	0.4	±	0.1	0.1	±	0.0
16:1ω7c	1.1	±	0.2	1.4	±	0.7
16:1ω13t	0.2	±	0.0	0.2	±	0.1
17:1ω8c	1.2	±	0.1	1.3	±	0.5
17:1	2.9	±	0.3	2.3	±	0.6
18:1ω9c	11.9	±	2.7	13.5	±	3.0
18:1ω7c	7.2	±	3.1	5.6	±	2.4
18:1ω7t	0.1	±	0.0	0.8	±	0.7
18:1ω5c	0.1	±	0.0	0.2	±	0.1
20:1ω9c	1.2	±	0.2	2.6	±	1.4
20:1ω7c	0.3	±	0.1	0.3	±	0.0
20:1ω5c	0.2	±	0.0	0.3	±	0.1
22:1ω11c	0.4	±	0.2	0.5	±	0.4
22:1ω9c	0.3	±	0.1	2.8	±	2.2
22:1ω7c	0.3	±	0.0	0.3	±	0.2
24:1ω11c	0.2	±	0.1	0.4	±	0.2
24:1ω9c	1.7	±	0.1	1.5	±	0.3
Total MUFA	**29.7**	±	**1.0**	**34.2**	±	**5.4**
18:3ω6	0.2	±	0.0	0.1	±	0.1
18:4ω3	0.0	±	0.0	0.6	±	0.4
18:2ω6	0.9	±	0.1	0.6	±	0.2
18:3ω3	0.2	±	0.1	2.6	±	2.2
20:4ω6	17.1	±	1.4	14.0	±	3.6
20:5ω3	1.7	±	0.2	1.6	±	0.4
20:3ω6	0.4	±	0.1	0.4	±	0.1
20:4ω3	0.4	±	0.2	1.0	±	0.4
20:2ω6	0.2	±	0.0	0.3	±	0.0
21:5ω3	0.1	±	0.0	0.2	±	0.1
22:5ω6	1.2	±	0.2	1.8	±	0.5
22:6ω3	3.4	±	0.6	3.1	±	1.2
22:4ω6	6.6	±	0.6	6.7	±	2.0
22:5ω3	3.3	±	0.4	2.3	±	0.6
Total PUFA	**35.8**	±	**2.1**	**35.4**	±	**3.9**
Others	0.3	±	0.0	0.3	±	0.0

Others (< 0.2%): i15:0, a15:0, i16:0, 14:1ω7c, 16:1ω7t, 16:1ω5c, 20:1ω11c
SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids
EPA: eicosapentanoeic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid
t: trans-configured MUFA, c: cis-configued MUFA
The suffix i denotes branched fatty acids from the iso-series. FALD: fatty aldehyde analysed as dimethyl acetal.

Appendix 2.2. The mean FA composition (% of total FA ± standard error) of potential prey collected at Ningaloo Reef in May 2013, 2014 and 2015.

Fatty acids	Fish larvae	Pelagic fish	*Spratelloides*	Myctophid	Cephalopods	Crab larvae
14:0	1.7 ± 0.2	1.4 ± 0.4	3.2 ± 0.9	2.7 ± 1.2	1.7 ± 0.4	2.5 ± 0.2
i15:0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
a15:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
15:0	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.3 ± 0.1
i16:0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.2
16:0	23.6 ± 0.8	24.7 ± 4.8	24.9 ± 1.1	25.5 ± 3.5	20.7 ± 2.0	19.5 ± 1.8
i17:0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.9 ± 0.7	0.3 ± 0.0	0.7 ± 0.1
17:0	1.6 ± 0.1	1.8 ± 0.5	1.9 ± 0.1	1.4 ± 0.1	2.5 ± 0.3	1.6 ± 0.1
i18:0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
18:0	11.8 ± 0.3	16.4 ± 4.6	10.5 ± 0.2	9.7 ± 1.9	9.7 ± 0.6	7.7 ± 0.6
20:0	0.5 ± 0.0	1.9 ± 0.9	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.2
22:0	0.5 ± 0.0	0.7 ± 0.4	0.5 ± 0.0	0.7 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
24:0	0.6 ± 0.1	1.3 ± 0.3	1.1 ± 0.1	0.9 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Total SFA	**41.8 ± 0.1**	**49.7 ± 0.5**	**44.8 ± 0.1**	**43.6 ± 0.3**	**37.3 ± 0.2**	**35.5 ± 0.1**
14:1ω7c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:1ω9c	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
16:1ω7c	2.9 ± 0.2	2.1 ± 0.5	3.7 ± 0.5	4.0 ± 0.4	1.8 ± 0.4	5.1 ± 0.3
16:1ω7t	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:1ω5c	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:1ω13t	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
17:1ω8c+a17:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.9 ± 0.4	0.5 ± 0.1	1.0 ± 0.2
17:1	0.7 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	1.0 ± 0.1	0.5 ± 0.1
18:1ω9c	7.5 ± 0.2	8.2 ± 0.7	7.1 ± 0.3	7.3 ± 1.3	6.1 ± 0.9	12.1 ± 1.0
18:1ω7c	2.6 ± 0.1	2.7 ± 0.1	3.0 ± 0.2	2.5 ± 0.3	2.3 ± 0.3	2.6 ± 0.2
18:1ω7t	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:1ω5c	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:1ω11c	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.1	0.8 ± 0.1	1.4 ± 0.1
20:1ω9c	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	2.1 ± 0.3	0.7 ± 0.1
20:1ω7c	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.1
20:1ω5c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
22:1ω11c	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
22:1ω9c	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.8 ± 0.1	0.1 ± 0.0
22:1ω7c	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
24:1ω11c	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
24:1ω9c	1.0 ± 0.1	1.7 ± 0.2	1.4 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	0.1 ± 0.0
Total MUFA	**17.1 ± 0.0**	**18.0 ± 0.0**	**18.5 ± 0.0**	**18.0 ± 0.1**	**17.4 ± 0.0**	**25.1 ± 0.0**
18:3ω6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1
18:4ω3	0.7 ± 0.1	0.4 ± 0.0	0.8 ± 0.0	1.1 ± 0.5	0.3 ± 0.1	1.0 ± 0.1
18:2ω6	1.2 ± 0.1	1.0 ± 0.3	1.4 ± 0.1	1.6 ± 0.3	0.8 ± 0.2	1.6 ± 0.1
18:3ω3	0.7 ± 0.1	0.3 ± 0.1	0.8 ± 0.0	0.8 ± 0.2	0.3 ± 0.1	0.7 ± 0.0
20:4ω6	2.8 ± 0.2	2.9 ± 1.0	3.0 ± 0.1	2.2 ± 0.5	4.6 ± 0.5	2.7 ± 0.1
20:5ω3	5.8 ± 0.2	3.3 ± 0.9	6.0 ± 0.2	6.6 ± 1.7	9.7 ± 0.5	10.6 ± 0.9
20:3ω6	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
20:4ω3	0.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.1
20:2ω6	0.3 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 0.1	1.4 ± 0.1
21:5ω3	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.0
22:5ω6	1.3 ± 0.3	4.3 ± 2.6	1.1 ± 0.1	1.8 ± 0.8	1.2 ± 0.1	1.9 ± 0.6
22:6ω3	24.6 ± 1.1	17.2 ± 5.8	20.1 ± 2.7	19.3 ± 5.5	23.4 ± 2.8	16.0 ± 1.4
22:4ω6	0.6 ± 0.0	0.7 ± 0.3	0.8 ± 0.1	3.2 ± 3.4	2.7 ± 1.5	0.5 ± 0.0
22:5ω3	2.1 ± 0.2	1.2 ± 0.4	1.3 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	1.5 ± 0.1
Total PUFA	**41.0 ± 0.1**	**32.4 ± 0.4**	**36.7 ± 0.2**	**38.4 ± 0.4**	**45.3 ± 0.2**	**39.4 ± 0.1**
w3/w6	**5.3 ± 0.3**	**2.4 ± 0.7**	**4.1 ± 0.3**	**3.2 ± 0.6**	**3.4 ± 0.6**	**3.5 ± 0.3**

Appendix 2.2. Continued

Fatty acids	Decapod	Isopods	Krill	Mysids	Amphipods	Annelida
14:0	1.9 ± 0.3	3.5 ± 2.3	1.0 ± 0.3	0.4 ± 0.1	0.5 ± 0.4	0.2 ± 0.1
i15:0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
a15:0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15:0	0.8 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.2	0.3 ± 0.1
i16:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:0	21.0 ± 0.9	23.9 ± 1.6	16.6 ± 1.3	17.9 ± 1.0	17.6 ± 11.5	17.4 ± 2.9
i17:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0
17:0	1.7 ± 0.1	0.8 ± 0.2	1.8 ± 0.0	1.2 ± 0.0	1.6 ± 0.5	2.5 ± 0.2
i18:0	0.3 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0
18:0	8.6 ± 0.4	5.3 ± 0.7	5.8 ± 0.2	6.8 ± 0.4	7.6 ± 2.6	16.7 ± 3.1
20:0	0.7 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.5 ± 0.2	0.3 ± 0.1
22:0	0.9 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.3 ± 0.2	0.5 ± 0.2
24:0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.5	0.4 ± 0.1
Total SFA	**36.8 ± 0.1**	**34.9 ± 0.2**	**27.4 ± 0.1**	**28.6 ± 0.1**	**29.7 ± 0.9**	**38.4 ± 0.3**
14:1ω7c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:1ω9c	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.4	0.0 ± 0.0
16:1ω7c	3.0 ± 0.2	2.3 ± 0.6	3.1 ± 0.3	1.8 ± 0.1	2.0 ± 1.9	0.7 ± 0.3
16:1ω7t	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:1ω5c	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.1 ± 0.1
16:1ω13t	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.2	0.2 ± 0.0
17:1ω8c+a17:0	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
17:1	0.5 ± 0.1	0.3 ± 0.0	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.3	4.9 ± 1.4
18:1ω9c	9.0 ± 0.7	11.3 ± 1.3	7.3 ± 0.2	10.8 ± 0.6	13.1 ± 11.1	7.5 ± 0.3
18:1ω7c	2.6 ± 0.2	1.6 ± 0.2	2.2 ± 0.1	3.2 ± 0.5	2.6 ± 1.2	2.9 ± 0.4
18:1ω7t	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
18:1ω5c	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
20:1ω11c	0.5 ± 0.0	0.4 ± 0.2	0.2 ± 0.0	0.7 ± 0.2	0.5 ± 0.3	2.7 ± 0.5
20:1ω9c	0.7 ± 0.1	0.8 ± 0.2	0.8 ± 0.0	1.0 ± 0.1	0.8 ± 0.3	0.3 ± 0.2
20:1ω7c	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
20:1ω5c	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
22:1ω11c	1.1 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.1
22:1ω9c	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.0 ± 0.0
22:1ω7c	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0
24:1ω11c	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1ω9c	0.8 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	0.2 ± 0.2
Total MUFA	**19.8 ± 0.0**	**18.3 ± 0.1**	**16.2 ± 0.0**	**20.1 ± 0.0**	**21.9 ± 0.5**	**20.4 ± 0.1**
18:3ω6	0.1 ± 0.0	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.7	0.0 ± 0.0
18:4ω3	0.5 ± 0.0	0.9 ± 0.5	0.7 ± 0.0	0.7 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
18:2ω6	1.5 ± 0.1	1.8 ± 0.6	2.3 ± 0.1	2.1 ± 0.0	1.1 ± 0.5	1.8 ± 0.2
18:3ω3	0.7 ± 0.1	1.1 ± 0.6	1.4 ± 0.0	1.4 ± 0.1	0.4 ± 0.3	0.5 ± 0.1
20:4ω6	4.0 ± 0.7	2.1 ± 0.6	5.2 ± 0.2	8.4 ± 0.8	8.5 ± 3.1	1.1 ± 0.2
20:5ω3	10.9 ± 0.5	13.5 ± 0.9	18.4 ± 0.8	15.5 ± 0.3	10.5 ± 4.7	18.4 ± 3.3
20:3ω6	0.2 ± 0.0	0.6 ± 0.3	0.2 ± 0.0	0.5 ± 0.0	0.6 ± 0.9	0.3 ± 0.1
20:4ω3	0.3 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.7 ± 0.2
20:2ω6	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	1.1 ± 0.1	0.5 ± 0.2	1.0 ± 0.1
21:5ω3	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
22:5ω6	2.6 ± 0.8	0.7 ± 0.2	1.3 ± 0.1	0.8 ± 0.0	1.5 ± 0.7	0.2 ± 0.1
22:6ω3	20.6 ± 1.2	22.3 ± 6.2	24.5 ± 0.5	17.5 ± 0.9	21.8 ± 10.9	1.8 ± 0.7
22:4ω6	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	1.2 ± 0.9	3.8 ± 0.6
22:5ω3	0.8 ± 0.1	1.8 ± 0.4	0.7 ± 0.1	1.7 ± 0.1	1.2 ± 0.7	11.4 ± 1.6
Total PUFA	**43.4 ± 0.1**	**46.8 ± 0.4**	**56.4 ± 0.1**	**51.3 ± 0.1**	**48.4 ± 0.8**	**41.2 ± 0.2**
w3/w6	**3.6 ± 0.7**	**4.0 ± 0.2**	**2.7 ± 0.2**	**2.4 ± 0.2**	**2.5 ± 0.6**	**1.5 ± 0.6**

Appendix 2.2. Continued

Fatty acids	Larger Zooplankton			*Sargassum*			Phytoplankton		
14:0	2.7	±	1.1	0.9	±	0.8	2.3	±	1.1
i15:0	0.5	±	0.1	0.1	±	0.0	0.1	±	0.1
a15:0	0.2	±	0.2	0.0	±	0.0	0.1	±	0.1
15:0	0.7	±	0.2	0.2	±	0.1	0.2	±	0.1
i16:0	0.2	±	0.1	0.2	±	0.2	0.1	±	0.0
16:0	22.2	±	4.2	24.8	±	8.8	22.5	±	3.1
i17:0	1.0	±	0.4	0.1	±	0.0	0.4	±	0.2
17:0	2.7	±	0.5	1.0	±	0.8	1.0	±	0.1
i18:0	0.6	±	0.2	0.0	±	0.0	0.1	±	0.0
18:0	16.7	±	6.2	5.5	±	4.0	10.6	±	1.5
20:0	1.1	±	0.3	0.7	±	0.2	1.3	±	0.2
22:0	1.0	±	0.3	0.7	±	0.2	0.7	±	0.2
24:0	0.8	±	0.3	0.4	±	0.1	1.3	±	0.2
Total SFA	**50.4**	**±**	**0.5**	**34.8**	**±**	**0.7**	**40.7**	**±**	**0.2**
14:1ω7c	0.0	±	0.0	0.0	±	0.0	0.1	±	0.1
16:1ω9c	1.3	±	0.5	0.0	±	0.0	0.4	±	0.2
16:1ω7c	3.1	±	1.3	1.9	±	1.0	4.8	±	1.3
16:1ω7t	0.2	±	0.2	0.0	±	0.0	0.0	±	0.0
16:1ω5c	0.1	±	0.0	0.1	±	0.1	0.0	±	0.0
16:1ω13t	0.1	±	0.0	0.7	±	0.3	0.3	±	0.1
17:1ω8c+a17:0	0.5	±	0.2	0.2	±	0.1	0.5	±	0.1
17:1	0.5	±	0.2	1.3	±	1.2	0.3	±	0.1
18:1ω9c	11.0	±	2.6	9.5	±	1.9	21.5	±	8.8
18:1ω7c	3.9	±	0.5	1.3	±	0.6	2.5	±	0.2
18:1ω7t	0.4	±	0.2	0.0	±	0.0	0.7	±	0.1
18:1ω5c	0.3	±	0.1	0.1	±	0.0	0.4	±	0.2
20:1ω11c	0.6	±	0.2	2.2	±	0.5	0.3	±	0.2
20:1ω9c	1.0	±	0.4	0.1	±	0.1	0.6	±	0.1
20:1ω7c	0.6	±	0.2	0.2	±	0.2	0.3	±	0.2
20:1ω5c	0.2	±	0.1	0.1	±	0.0	0.2	±	0.1
22:1ω11c	0.7	±	0.5	0.9	±	0.4	0.1	±	0.0
22:1ω9c	0.5	±	0.2	0.1	±	0.0	0.3	±	0.2
22:1ω7c	0.2	±	0.1	0.1	±	0.1	0.1	±	0.1
24:1ω11c	0.1	±	0.0	0.2	±	0.1	0.2	±	0.1
24:1ω9c	0.8	±	0.3	0.0	±	0.0	0.3	±	0.1
Total MUFA	**26.1**	**±**	**0.1**	**18.9**	**±**	**0.1**	**34.0**	**±**	**0.4**
18:3ω6	0.2	±	0.1	0.5	±	0.2	1.8	±	0.4
18:4ω3	0.8	±	0.3	3.7	±	2.2	2.3	±	0.5
18:2ω6	1.8	±	0.4	4.6	±	1.5	5.2	±	1.3
18:3ω3	0.8	±	0.2	4.5	±	2.5	1.9	±	0.3
20:4ω6	2.4	±	0.7	9.5	±	3.5	0.8	±	0.2
20:5ω3	5.9	±	2.0	12.7	±	8.1	3.2	±	0.7
20:3ω6	0.4	±	0.1	0.8	±	0.0	0.2	±	0.1
20:4ω3	0.6	±	0.2	0.7	±	0.5	0.4	±	0.2
20:2ω6	0.3	±	0.1	0.8	±	0.4	0.7	±	0.2
21:5ω3	0.6	±	0.4	0.0	±	0.0	0.2	±	0.1
22:5ω6	1.1	±	0.4	0.1	±	0.1	0.7	±	0.2
22:6ω3	6.9	±	2.0	0.3	±	0.2	6.9	±	1.9
22:4ω6	0.5	±	0.2	2.6	±	2.5	0.4	±	0.1
22:5ω3	1.2	±	0.8	5.5	±	5.5	0.7	±	0.2
Total PUFA	**23.5**	**±**	**0.2**	**46.3**	**±**	**0.6**	**25.4**	**±**	**0.1**
w3/w6	**4.6**	**±**	**0.1**	**2.5**	**±**	**0.4**	**1.6**	**±**	**0.4**

Other minor FA in samples include: i14:0, 14:1ω5c, 15:1ω6c, 16:4, 16:3, 16:0FALD, C18PUFA, 18:1, 18:1FALD, 18:0FALD, 19:1A, 19:1B, 19:0, 21:0, C22PUFA, 23:0, 24:6ω3, 24:5ω3, 24:1ω7c.

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid

t: trans-configured MUFA, c: cis-configured

The sufix i denotes branched fatty acids from the iso-series. FALD: fatty aldehyde analysed as dimethyl acetal.

Appendix 2.3. Lipid content (mg g^{-1} dm) and lipid class composition (% of total lipids ± standard error) for potential prey collected at Ningaloo Reef in May 2013, 2014 and 2015.

Parameter			Fish larvae (n = 19)	Pelagic fish (n = 3)	Cephalopods (n = 4)	*Spratelloides* sp (n = 1)	Crab larvae (n = 7)
Lipid content	$mg.g^{-1}$	dm	28.8 ± 6.0	41.9 ± 13.3	117.7 ± 25.5	34.3 ± -	115.1 ± 36.2
Lipid class composition							
Wax esters	%		7.6 ± 1.4	2.8 ± 1.5	1.7 ± 0.7	2.1 ± -	0.9 ± 0.5
Triacylglycerols	%		12.2 ± 2.2	11.0 ± 3.1	8.3 ± 4.9	22.9 ± -	72.7 ± 9.3
Free fatty acids	%		12.2 ± 3.5	5.4 ± 3.5	4.6 ± 0.9	2.9 ± -	1.9 ± 0.3
Sterols	%		6.5 ± 0.8	5.5 ± 1.5	13.8 ± 2.9	7.8 ± -	1.9 ± 0.5
Phospholipids	%		57.0 ± 4.5	75.4 ± 6.0	71.6 ± 5.3	64.3 ± -	22.6 ± 8.9

Parameter			Decapods (n = 7)	Myctophid (n = 1)	Isopods (n = 2)	Annelida (n = 2)	*Sargassum* sp (n = 1)
Lipid content	$mg.g^{-1}$	dm	80.4 ± 43.7	62.9 ± -	106.9 ± 0.0	27.7 ± 4.9	17.1 ± -
Lipid class composition							
Wax esters	%		2.1 ± 0.9	1.7 ± -	7.6 ± 0.7	9.6 ± 3.5	2.2 ± -
Triacylglycerols	%		5.2 ± 2.2	7.5 ± -	43.7 ± 1.3	1.3 ± 1.3	3.3 ± -
Free fatty acids	%		3.3 ± 1.0	6.6 ± -	8.6 ± 0.9	2.2 ± 2.2	2.9 ± -
Sterols	%		7.9 ± 1.2	12.4 ± -	2.5 ± 0.2	11.1 ± 0.0	6.4 ± -
Phospholipids	%		82.1 ± 3.3	71.8 ± -	37.5 ± 2.7	75.8 ± 0.1	85.2 ± -

Parameter			Larger Zooplankton (n = 2)	Krill (n = 5)	Mysids (n = 7)	Amphipods (n = 2)
Lipid content	$mg.g^{-1}$	dm	9.7 ± 9.0	28.8 ± 5.9	38.2 ± 2.0	51.5 ± 2.8
Lipid class composition						
Wax esters	%		8.8 ± 8.8	2.2 ± 1.0	3.9 ± 1.4	3.9 ± 2.8
Triacylglycerols	%		0.5 ± 0.5	2.7 ± 1.2	21.1 ± 7.9	28.8 ± 10.1
Free fatty acids	%		1.2 ± 1.2	2.4 ± 1.0	15.9 ± 8.0	7.0 ± 4.5
Sterols	%		15.0 ± 5.5	10.4 ± 0.9	9.9 ± 1.6	7.5 ± 0.4
Phospholipids	%		74.5 ± 1.7	82.4 ± 2.6	49.1 ± 8.8	52.8 ± 3.2

Appendix 3.1. Description of samples included in chapter 3.

Taxa		n	Tissue type	Year of sampling	Habitat
Elasmobranch					
	Whale shark *(Rhincodon typus*)	15	Sub-dermal	2013 - 2014	Tropical
Parasitic copepods					
	Pandarus rhincodonicus	21	Whole	2013 - 2014	Tropical
Zooplankton[a]		11	Whole	2013	
	Calanoid copepod *(Paracalanus sp.)*				
	Cyclopoid copepod *(Oncaea sp.)*				
	Cyclopoid copepod *(Farranula sp.)*				
	Chaetognath (Sagittidae)				
	Chaetognath (Eukrohniidae)				
	Ostracoda				
	Foraminifera				
	Gastropoda				
	Doliolid				
	Amphipoda (Hyperiidea)				
	Amphipoda (Gammaridea)				
	Fish larvae				
	Echinoderm larvae				
	Decapod larvae				
Mysids					
	Heteromysinae	76	Whole	2014	Tropical
Euphausiids					
	Euphausiidae *(Thysanopoda tricuspidata)*	220	Whole	2014	Tropical
Decapods					
	Crab larvae (Eurachyura)	29	Whole	2014	Tropical
	Mantis shrimp (Stomatopoda)	5	Whole	2014	Tropical
Cephalopods					
	Octopus larvae	7	Whole	2014	Tropical
Fish larvae					
	Sabre toothed blenny (*Aspidontus taeniatus*)	6	Whole	2014	Tropical
	Rainbow runner (*Elagatis bipinnulata*)	4	Whole	2014	Tropical
	Goat fish (Mullidae)	5	Whole	2014	Tropical
	Emperor *(Lethrinus sp.*)	11	Whole	2014	Tropical
	Cardinal fish (*Apogonidae sp.*)	10	Whole	2014	Tropical

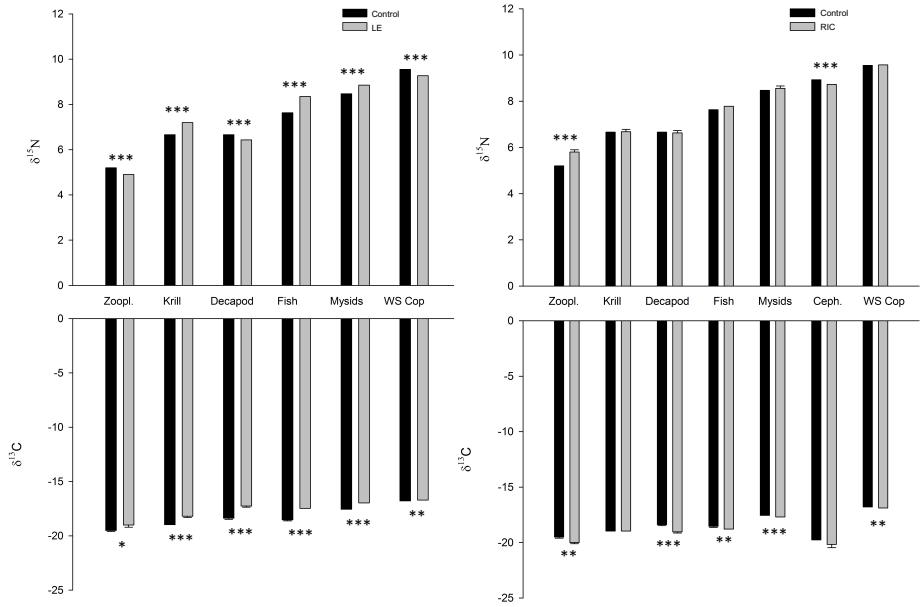
[a] Zooplankton samples were a mix of species and are listed in decreasing order of abundance

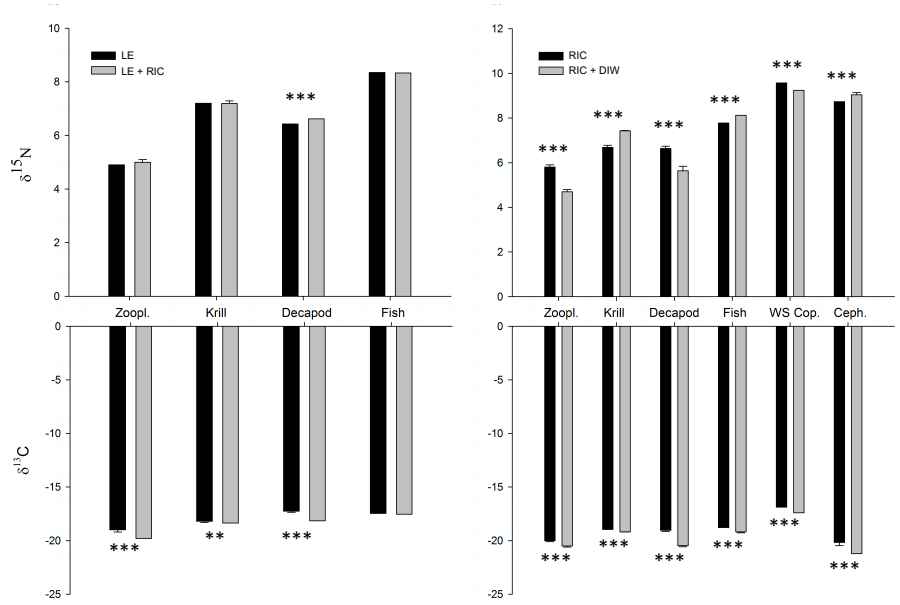
Appendix 3.2. One-way ANOVA test or GLS model comparing δ13C and δ15N values and C:N ratios of zooplankton and micronekton taxa among different treatments: untreated (Control), lipid extracted (LE), acidification (RIC), lipid extraction combined with acidification (LE+RIC), acidification combined with deionized water rinsing (RIC+DIW).

		One-way ANOVA test or Generalised Least Square model (GLS)							
Taxa	**Variables**	**Control vs LE**	**Control vs RIC**	**Control vs LE+RIC**	**Control vs RIC+DIW**	**LE vs RIC**	**LE vs LE+RIC**	**RIC vs LE+RIC**	**RIC vs RIC+DIW**
Zooplankton	δ13C	*	**	p = 0.123	***	***	***	*	***
	δ15N	***	***	*	***	***	p = 0.900	***	***
	C:N	***	***	***	***	***	***	***	***
Euphausiids	δ13C	***	p = 0.992	***	***	***	**	***	***
	δ15N	***	p = 1	***	***	***	p = 0.999	***	***
	C:N	***	p = 0.580	***	p = 0.343	p = 1	p = 0.367	p = 1	p = 0.453
Decapods	δ13C	***	***	*	***	***	***	***	***
	δ15N	***	p = 0.992	p = 0.840	***	p = 0.341	***	p = 0.999	p = 0.261
	C:N	***	p = 0.934	***	***	***	***	***	***
Fish larvae	δ13C	***	**	***	***	***	p = 0.437	***	**
	δ15N	***	p = 0.650	***	***	***	p = 0.989	***	***
	C:N	***	p = 0.890	***	***	***	p = 0.999	***	***
Mysids	δ13C	***	***	NA	NA	***	NA	NA	NA
	δ15N	***	p = 0.340	NA	NA	***	NA	NA	NA
	C:N	***	**	NA	NA	***	NA	NA	NA
Cephalopods	δ13C	NA	p = 0.404	NA	***	NA	NA	NA	***
	δ15N	NA	***	NA	p = 0.21	NA	NA	NA	***
	C:N	NA	p = 0.821	NA	***	NA	NA	NA	***
Parasitic copepods	δ13C	**	**	NA	***	***	NA	NA	***
	δ15N	***	p = 0.922	NA	***	***	NA	NA	***
	C:N	***	**	NA	***	***	NA	NA	***

Statistical significance: * p < 0.05, ** p < 0.01, *** p< 0.001
NA: no data for these treatments

Appendix 3.3. Comparison of $\delta^{13}C$ and $\delta^{15}N$ values (‰) among treatments for zooplankton and micronekton taxa (krill, decapods, fish larvae, mysids, cephalopods and whale shark parasitic copepods): untreated (Control), lipid extracted (LE), acidification (RIC), lipid extraction combined with acidification (LE+RIC), acidification combined with deionized water rinsing (RIC+DIW). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ according to one-way ANOVA test and GLS model.





Appendix 4.1. Biological information of the individual whale sharks sampled at Ningaloo Reef and results of stable isotope analysis (δ13C and δ15N values and C:N ratios) in 2013 and 2014. M = male, F = female.

ID	Year of collection	Sex	Total Length (m)	$\delta^{13}C$	$\delta^{15}N$	C:N
1	2013	M	3.5	-15.4	9.1	2.6
2	2013	M	4.5	-14.9	6.9	2.8
3	2013	M	5.5	-14.4	8.8	2.7
4	2013	F	4	-15.3	7.8	2.9
5	2013	M	6.5	-15.7	9.0	2.8
6	2013	F	4	-15.5	8.1	2.9
7	2013	M	5	-15.6	8.5	2.9
8	2013	M	6	-15.4	8.8	3.0
9	2013	M	5	-15.2	8.7	2.9
10	2013	M	5	-14.4	8.5	2.8
11	2013	M	7.5	-13.3	9.9	2.8
12	2013	M	7	-14.9	10.7	2.9
13	2013	M	5.5	-15.5	10.3	2.9
14	2013	F	6.5	-15.7	10.0	2.9
15	2013	F	6	-15.2	9.5	2.8
16	2013	M	6	-15.0	9.4	2.9
17	2013	M	7.5	-13.3	10.8	2.7
18	2013	M	4.5	-15.3	10.6	2.8
19	2013	F	6	-15.1	9.9	2.9
20	2013	M	5.5	-15.5	9.8	3.0
21	2013	M	7	-14.8	10.5	2.8
22	2013	M	5.5	-15.0	10.2	2.9
23	2013	M	3	-15.5	9.1	3.1
24	2013	M	8.5	-15.7	9.8	3.0
25	2014	M	3	-14.7	7.0	3.0
26	2014	M	5	-15.3	8.7	2.9
27	2014	F	3	-14.8	6.9	2.9
28	2014	M	5.5	-14.0	8.5	2.9
29	2014	M	4	-14.9	8.8	2.9
30	2014	M	7	-15.4	9.2	2.7
31	2014	F	5	-15.6	9.0	2.9
32	2014	M	6	-15.1	8.9	2.9
33	2014	F	6	-15.7	10.0	3.0
34	2014	M	5	-15.3	9.6	2.9
35	2014	M	4	-15.0	10.3	2.9
36	2014	M	4	-15.4	10.4	2.9
37	2014	M	8	-14.8	9.7	2.8
38	2014	M	7	-15.4	9.4	2.9
39	2014	M	5	-15.2	8.8	2.8
40	2014	M	8	-14.3	9.4	2.7
41	2014	M	8	-14.5	9.5	2.7
42	2014	M	5	-14.7	7.3	2.9
43	2014	F	5	-14.8	9.5	2.8
44	2014	M	5.5	-15.2	8.5	2.9
45	2014	M	4.5	-15.1	9.2	2.9
46	2014	M	4	-15.7	8.9	3.0
47	2014	F	4.5	-14.3	8.9	2.9
48	2014	M	4.5	-14.1	10.0	2.8
49	2014	M	6	-14.7	8.3	2.8
50	2014	F	7	-14.0	10.2	2.8